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Bone Marrow Appearances in Reticulum Cell Sarcoma

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Key Words Bone marrow biopsy Malignant histiocytic lymphoma Reticulum cell sarcoma

Abstract 18 patients with malignant lymphoma histiocytic type were investigated for the bone marrow involvement Bone marrow aspirates and needle biopsies were used Smears and sections of the aspirates were examined The findings in the sections of both aspirates and biopsies were complementary and far more valuable than the bone marrow smears alone It is concluded that bone marrow examination is helpful in determining the spread of the disease and should be repeated during the course of lymphoma

The diagnosis of the reticulum cell sarcoma (malignant histiocytic lymphoma) is not usually made from the examination of a bone marrow smear Further it has been suggested [1] that bone marrow biopsy is unsatisfactory in this relatively uncommon condition In the last 2 years we have had the opportunity of studying the bone marrow of 18 patients with a diagnosis of malignant histiocytic lymphoma In every case the diagnosis was made by histological examination of the involved tissue or lymph node biopsy

Early in this study examination of sections from a bone marrow biopsy revealed the presence of abnormal polygonal cells resembling reticulum cells which were *not present in the smears This suggested that* the abnormal cells were more difficult to aspirate or were distorted or destroyed during spreading In an attempt to examine these possibilities it was decided to compare the results of aspiration with that obtained by histological examination of biopsy material

Patients

18 patients who presented themselves between 1971 and 1973 were studied. There were equal numbers of males and females and their ages ranged from 42 to 74 years. Brief clinical and haematological findings are presented in table I.

A bone marrow aspirate and biopsy material were obtained from the same site at the time of diagnosis and subsequently as indicated by the clinical and haematological findings. In some instances both procedures could not be carried out.

Methods

The marrow cavity was entered using a modified Waterfield needle, and the aspirate was divided into two: one was used to prepare films and the other was placed whole in 10% buffered formal saline (pH 7.0) and subsequently prepared for histological examination. Bone marrow biopsy was performed using a modified Gardner's needle and the specimen was placed immediately in 10% buffered formaline and held for 12 h and then processed using routine histological techniques. The bone marrow films were stained with Jenner Giemsa stain. The histological section was stained with Giemsa [5], haematoxylin and eosin, and for reticulin [2]. Six films of the bone marrow aspirate and representative histological sections of aspirates and biopsies were read independently by at least two observers.

Results

A total of 24 aspirations and 13 biopsies were obtained from the 18 patients included in this series. Examination of representative smears from the 24 aspirates showed the presence of abnormal histiocyte (reticulum) cells in only 5 instances (table II). These cells were scanty in number (<5% of the total nucleated cells) and tended to be scattered throughout the smear. Patient No 3 not only had these immature cells in the bone marrow but a few morphologically similar cells were seen in the peripheral blood. The disease was widespread at this time and the patient died a few months later.

Histological section of the aspirate. 16 specimens from 18 patients were available for histological examination. The overall structure and cellularity of the marrow was more clearly demonstrated than in the aspirates. Of 16 samples 3 showed marked infiltration (>5% of the total nucleated cells present) with immature histiocytes (table II). Figure 1 indicates the appearances in these sections. The large polygonal cells of

Table 1 Clinical features and haematological findings at the time of presentation in 18 patients with malignant lymphoma 'histiocytic type'

Case No	Age years	Sex	Clinical features	Hb g%	WBC µl	Plats µl	Site of histological diagnosis
1	62	m	inguinal lymphadenopathy, hepatosplenomegaly	9.6	22,100	400 000	lymph node
2	61	m	cervical lymphadenopathy followed by inguinal lymph node enlargement, no hepatosplenomegaly	12.1	2,200	125 000	lymph node
3	[illegible]	m	cervical lymphadenopathy followed by a mass in the suprapubic area and enlarged inguinal lymph nodes, no hepatosplenomegaly	10.1	8 900	256 000	tonsils
4	[illegible]	f	painful swelling of the right knee, inguinal lymphadenopathy, hepatomegaly, no splenomegaly	9.0	7 [illegible]00	134 000	lymph node
5	64	f	Large abdominal mass, ascites, hepatomegaly no splenomegaly	11.5	6,700	225,000	abdominal tumour
6	[illegible]	m	enlarged cervical lymph nodes for 2 months no hepatosplenomegaly	9.5	2,200	137 000	lymph node
7	64	f	cervical lymphadenopathy for 5 months, generally unwell no hepatosplenomegaly	12.1	3,500	161,000	lymph node
8	[illegible]	f	cervical lymphadenopathy followed by generalised lymphadenopathy, abdominal mass, hepatomegaly no splenomegaly	11.5	4,200	256,000	lymph node
9	67	f	loss of weight, anaemia hepatosplenomegaly, generalised lymphadenopathy	9.5	9 100	normal	lymph node
10	42	m	right submaxillary mass followed by cervical and inguinal lymphadenopathy	13.4	7 000	174 000	lymph node
11	41	m	polycystic kidneys, chronic renal failure (on dialysis twice weekly). Pyrexia for a few months, followed by enlargement of axillary nodes no hepatosplenomegaly	9.5	4,200	normal	lymph node

Patients

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2	61	m	cervical lymphadenopathy followed by inguinal lymph node enlargement, no hepatosplenomegaly	12 1	2,200	128 000	lymph node
3	69	m	cervical lymphadenopathy followed by a mass in the suprapubic area and enlarged inguinal lymph nodes, no hepatosplenomegaly	10 1	8,900	256,000	tonsils
4	56	f	painful swelling of the right knee, inguinal lymphadenopathy, hepatomegaly, no splenomegaly	9 0	7,600	134,000	lymph node
5	64	f	Large abdominal mass, ascites, hepatomegaly, no splenomegaly	11 5	6,700	225,000	abdominal tumour
6	69	m	enlarged cervical lymph nodes for 2 months, no hepatosplenomegaly	9 5	2,200	137,000	lymph node
7	64	f	cervical lymphadenopathy for 5 months, generally unwell, no hepatosplenomegaly	12 3	3,500	161,000	lymph node
8	59	f	cervical lymphadenopathy followed by generalised lymphadenopathy, abdominal mass, hepatomegaly no splenomegaly	11 5	4,200	286,000	lymph node
9	67	f	loss of weight, anaemia, hepatosplenomegaly, generalised lymphadenopathy	9 5	9,100	normal	lymph node
10	42	m	right submaxillary mass followed by cervical and inguinal lymphadenopathy	13 4	7,000	174,000	lymph node
11	45	m	polycystic kidney, chronic renal failure (on dialysis twice weekly) Pyrexia for a few months, followed by enlargement of axillary nodes no hepatosplenomegaly	9 5	4,200	normal	lymph node

Table I (continued)

Case No	Age years	Sex	Clinical features	Hb g%	WBC µl	Plats µl	Site of histological diagnosis
12	58	f	2 years history of enlarged left parotid gland, then inguinal lymphadenopathy, hepatomegaly, no splenomegaly	11 5	7,400	330 000	parotid gland
13	69	f	abdominal pain, nausea, vomiting for 1 year, partial gastrectomy years previously; no lymphadenopathy, no hepatosplenomegaly	4 1	2,8000	246 000	parietal gastric tumour
14	80	m	enlarged axillary lymph nodes, otherwise well, hepatomegaly, no splenomegaly	11 7	8,900	248 000	lymph node
15	71	f	cervical lymphadenopathy, no hepatosplenomegaly	13 2	5,400	199,000	lymph node
16	70	m	painless swelling of the left testis, no hepatosplenomegaly	12 9	5,700	108 000	testicular tumour
17	73	m	loss of weight over 1 year, chest X-ray enlarged paratracheal nodes, no hepatosplenomegaly	11 9	6 000	91,000	lymph node
18	73	m	left axillary lymphadenopathy followed by enlargement of right parotid gland and supraclavicular lymph node	14 1	7,000	normal	lymph node

15–30 µm in diameter are present with open nuclear chromatin containing 1–3 nucleoli These are the neoplastic histiocytic cells In 5 other instances only small numbers (less than 5%) of these immature cells were seen Significantly greater numbers of abnormal cells could be identified in the sections when compared with the marrow smears A positive correlation was found between the sections and smears when many primitive cells were present but the sections revealed clear evidence of the disease when this could not be demonstrated in the smears (cases 1, 2 and 8) even when these were studied later in a retrospective analysis

Histological section of the biopsies Apart from the greater opportunity to estimate the bone marrow cellularity 3 of 13 (23%) were found to

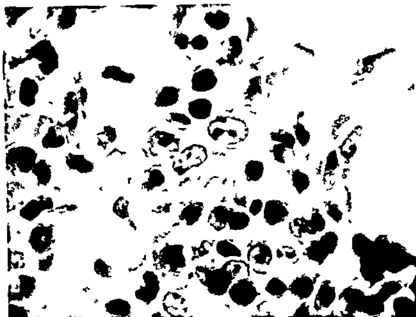


Fig 1 Section of a bone marrow aspirate many large immature reticulin cells with irregular nuclei and open chromatin pattern are scattered through the field HE × 960

contain many immature histiocytic cells There were also 5 of 13 (38%) in which the abnormal cells were present in small numbers (table II)

Correlation between the appearance of the biopsies and aspirates (table III) Where satisfactory specimens were available the findings in the biopsies correlated well with the section of the marrow aspirate In patient No 4 only occasional cells were present in the aspirate sections while they were more numerous in the biopsy specimen A higher percentage of positives was achieved using biopsy material and the marrow architecture and the quantity of reticulin could be assessed more accurately

Reticulin staining Increased amounts of reticulin material were present in 2 of the 8 with abnormal cells present A marked increase was only found in one instance when many primitive cells were present

Table II Appearance of bone marrow aspirates and biopsies

Case No	Film of aspirate	Histologic section of aspirate	Histologic section of biopsy
1	increased cellularity ME ratio 4:1 normal marrow	increased cellularity, immature histiocytic cells, < 5%[1]	normal cellularity, immature histiocytic cells ~5%[1], reticulin normal
	normal cellularity ME ratio 4:1 normal marrow	unsatisfactory	normal cellularity, normal erythro- and granulopoiesis, no abnormal cells, reticulin normal
2	normal cellularity ME ratio 1.5:1 normal marrow	cellular marrow, depressed erythropoiesis and myelopoiesis, immature histiocytic cells, < 5%[1]	unsatisfactory
	normal cellularity ME ratio 1:1 normal marrow	insufficient	normal cellularity, normal marrow, reticulin slightly increased
3	decreased cellularity ME ratio 1.4:1 normal marrow	hypocellular marrow, no abnormal cells	normal cellularity, occasional primitive cell, < 5%, reticulin normal
	normal cellularity ME ratio 1.4:1 immature histiocytic cells, < 5%[1]	normal cellularity, normal erythro- and myelopoiesis, immature histiocytic cells, < 5%[1]	irregular cellularity, many immature histiocytic cells > 5%[1], marked increase in reticulin
4	normal cellularity ME ratio 0.7:1 prominent plasma cells, otherwise normal marrow	normal marrow atypical immature cells, 5%[1]	normal cellularity, early granulocytes prominent immature histiocytic cells, 5%[1], reticulin normal
	normal cellularity ME ratio 2.8:1 normal marrow	insufficient	fragments of bone showing many large immature histiocytic cells, 5%[1], reticulin slightly increased
5	decreased cellularity ME ratio 2:1 hypocellular marrow	hypocellular marrow	scanty marrow immature histiocytic cells, 5%[1] reticulin normal
6	normal cellularity ME ratio 1.5:1 normal marrow	unsatisfactory	increased cellularity many immature histiocytic cells > 5%[1], reticulin normal

Table II (continued)

Case No	Film of aspirate	Histologic section of aspirate	Histologic section of biopsy
7	normal cellularity ME ratio 2:1 immature histiocytic cells, 5%[1]	insufficient	not done
8	normal cellularity ME ratio 2:1 immature histiocytic cells, < 5%[1]	very cellular marrow infiltrated with immature histiocytic cells, >5%[1]	not done
	normal cellularity ME ratio 3.6:1 normal marrow	hypercellular marrow normal erythro- and myelopoiesis, immature histiocytic cells, < 5%[1]	not done
	normal cellularity ME ratio 3.6:1 normal marrow	*few cellular areas* normal marrow	not done
9	normal cellularity ME ratio 4:1 immature histiocytic cells, < 5%[1]	cellular marrow infiltrated by large primitive histiocytic cells, >5%[1]	not done
10	normal cellularity ME ratio 3:1 normal marrow	normal cellularity normal marrow	fragments of cellular marrow, no abnormal cells, reticulin normal
11	increased cellularity ME ratio 2:1 normal marrow	normal cellularity, normal marrow	not done
12	normal cellularity ME ratio 4:1 few atypical immature cells and some smear cells	markedly cellular marrow, many large polygonal immature histiocytic cells, 5%[1]	not done
13	decreased cellularity ME ratio 1.4:1 normal marrow	scanty fragments normal marrow	unsatisfactory
14	normal cellularity ME ratio 4:1 normal marrow	unsatisfactory	deferred

Table II (continued)

Case No	Film of aspirate	Histologic section of aspirate	Histologic section of biopsy
15	normal cellularity M/E ratio 4:1 normal marrow	moderately cellular marrow; no histological abnormality	unsatisfactory
16	decreased cellularity M/E ratio 1.3:1 normal marrow	unsatisfactory	normal cellularity, normal erythro- and myelopoieses, no abnormal cells, reticulin normal
17	normal cellularity M/E ratio 8:1 dyserythropoiesis	normal cellularity, normal marrow	unsatisfactory
18	normal cellularity M/E ratio 7:1 immature histiocytic cells, <5%[1]	unsatisfactory	unsatisfactory

[1] >5% = Considered positive evidence of infiltration. <5% = Suspicious of infiltration

Discussion

In malignant histiocytic lymphoma, the bone marrow involvement is rarely verified by aspiration or biopsy [1]. In a large series of patients with a variety of lymphoma, bone marrow was examined at the time of diagnosis [3]. Only one positive sample was observed in 19 bone marrows from patients with untreated histiocytic lymphoma.

The superiority of histologic sections of aspirated bone marrow for diagnostic purposes in malignant lymphoma has been shown by LIAO [4]: 27 aspirates were positive using the sections but only 9 of these showed evidence of the disease on the smears. The findings presented here in malignant histiocytic lymphoma also reveal this discrepancy between the smears and the sections of the marrow aspirate. It seems likely that the malignant cells are fragile so that spreading disrupts or distorts many of them. Looking at the smears it is often difficult to determine the origin of some of the distorted or 'bald' cells which might well be histiocytic in origin. Foci of abnormal cells become dispersed through

Table III Correlation between aspirates and biopsies of bone marrow

Case No	Aspirate Film	Section	Biopsy	Reticulin
1	N	<5%	<5%	N
	N	–	N	N
2	N	<5%	–	–
	N	–	N	slightly increased
3	N	N	<5%	N
	<5%	<5%	>5%	markedly increased
4	N	<5%	<5%	N
	N	–	>5%	slightly increased
5	N	N	<5%	N
6	N	–	>5%	N
7	<5%		–	–
8	<5%	5%		–
	N	<5%		–
9	<5%	>5%		–
12	N	>5%		–
16	N	–	<5%	N
18	<5%			

>5% = Considered positive evidence of infiltration <5% = suspicious of infiltration
N = no increase of primitive histiocytic cells or reticulin

the film and are often difficult to recognise For these reasons it is helpful to study sections of an aspirate whenever a malignant lymphoma is suspected A difficulty arises in that a scanty aspirate is sometimes obtained probably due to an increase in the reticulin thus there may be insufficient material for the preparation of suitable sections A needle biopsy is more likely to provide adequate material to study the marrow architecture and the amount of reticulin present This procedure is simple and can be carried out under local anaesthesia at the same time as the aspirate is obtained The greater amount of tissue makes a diagnosis more likely providing the specimen is not distorted

Staining for reticulin was only of limited value and the condition could not be excluded even when it is not increased It has been suggested that pronounced fibrosis in malignant lymphomas of the histiocytic type is associated with a less aggressive illness and a slower rate of

growth [6] We found a marked increase in reticulin in only one instance when the patient had widespread disease which advanced rapidly.

Using current chemotherapeutic regimes, patients with malignant histiocytic lymphoma have a much improved outlook, so that it becomes important to search for possible recrudescence of this condition during the course of the disease. Repeated biopsies should be performed and in our experience are helpful in determining when the condition is becoming widespread. More active measures are indicated once primitive cells are found in the marrow as this demonstrates the widespread nature of the disease. The effectiveness of the subsequent therapy can be monitored by repeated bone marrow examination

Acknowledgements Sections of bone marrows were prepared in the Department of Histopathology at Charing Cross Hospital Medical School We would like to thank Dr B Fox, Dr J G JACKSON and Dr I. J PARADINAS of the same Department who kindly allowed us to have access to records and freedom to study and use some of their material

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Erythroblastic Islands and Ineffective Erythropoiesis in Acute Myeloid Leukaemia

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Key Words Anaemia in leukaemia Erythroblastic islands Erythropoiesis Leukaemia

Abstract The erythropoietic part of the bone marrow has been morphologically analysed in 34 untreated, anaemic patients with acute myeloid leukaemia and in 17 healthy controls In the patients with leukaemia the percentage of basophilic erythroblasts was abnormally high and elevated mitotic indices of the erythroblasts were recorded The accumulation of basophilic erythroblasts and a high frequency of megaloblastoid changes of the cells may indicate ineffective erythropoiesis A threefold increase of 'erythroblastic islands', i e erythroblasts in contact with reticulum cells were recorded in the patients with erythroleukaemia and a twofold increase in the patients with acute myeloid leukaemia The erythroblastic islands may indicate phagocytosis of defective erythroblasts and an intramedullary haemolysis may thus contribute to the development of anaemia

Anaemia is a regular complication in acute myeloid leukaemia In some cases, bleeding tendency or haemolysis is a prominent symptom and a reduced amount of erythropoietic tissue due to invasion by leukaemic granulopoietic cells in the bone marrow may sometimes contribute to anaemia [6, 18]

As early as 1933, Jaffé [14] stressed that replacement of red cell precursors by leukaemic cells could not satisfactorily explain the anaemia He also suggested that the erythropoietic cells are engaged in the leukaemic process Recent cytogenetic studies have shown evidence of leukaemic involvement of the erythroblasts not only in erythroleukaemia (EL) but also in cases of acute granulocytic leukaemia [2, 15, 16] Results of kinetic investigations in acute leukaemia suggest that an ineffective erythropoiesis due to a defective maturation of the erythroblasts may be of importance to the development of anaemia [9, 13, 17]

The aim of the present work was to investigate whether any characteristic changes in composition, morphology and mitotic activity of the erythropoietic pool will occur in different forms of acute myeloid leukaemia.

Material and Methods

Patients. 34 untreated consecutive patients with acute myeloid leukaemia were investigated at the time of diagnosis. In order to permit studies of the erythropoiesis only patients with more than 2% erythroblasts in their bone marrow smears were included. Nine suffered from EL, 20 from acute myeloblastic leukaemia (AML) and 5 from acute myelomonocytic leukaemia (AMML). The age of the patients was 7–90 years (mean 59). There were 19 men and 15 women. The age and sex distribution were comparable to those in more comprehensive investigations [10].

EL. The mean survival time after diagnosis was 3.6 months (0.4–9.9) and finally all patients developed an accumulation of myeloblasts in the bone marrow within 0.3–2.3 months. All patients had erythroblasts and granulopoietic precursor cells in their peripheral blood. In the bone marrow the percentage of erythroblasts varied between 25 and 90.

AML+AMML. 24 patients have died with a mean survival time of 5.2 months after diagnosis. The erythropoietic part of the bone marrow comprised 3–26% of the cells.

Controls. 17 apparently healthy persons, 7 men and 10 women, with normal haematological data and normal ESR served as controls. Their age was 19–82 years (mean 51).

Bone marrow examination. The bone marrow smears were stained with May Grunwald Giemsa. Through examination of 1,000 nucleated bone marrow cells the percentage of erythroid precursors was determined. 763–2,000 erythroblasts (mean 1,144) were then examined and classified according to HEILMEYER and BEGEMANN [12]. Proerythroblasts and basophilic erythroblasts were pooled into one group and denominated basophilic erythroblasts. A mitotic index of the erythroblasts was also calculated. The proportion of erythroblasts with a megaloblastic morphology was determined. When erythroblasts were found in contact with or apparently lying within a reticulum cell the formation was classified as an erythroblastic island and the number of such formations per 1,000 erythroblasts was registered.

Statistics. The results are expressed as mean values. Student's t test was used to estimate significance.

Results

As expected, the Hb concentrations were significantly lower in the patient group compared to the controls. In the EL group the Hb con-

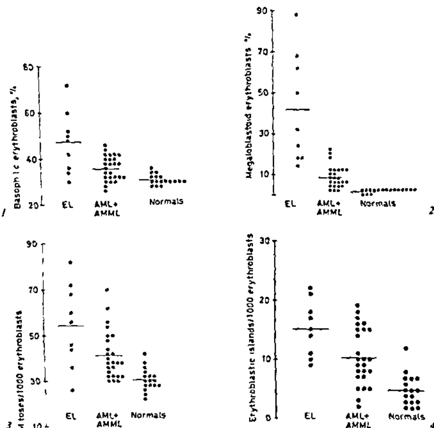


Fig 1 Proportions of basophilic erythroblasts within the erythropoietic series
Fig 2 Percentage of erythroblasts with megaloblastoid changes
Fig 3 Mitotic indices of the erythroblasts
Fig 4 Number of erythroblastic islands per 1 000 erythroblasts

centrations in g/100 ml were 4 0–7 9 (mean 5 8), in the AML+AMML group 4 0–12 4 (mean 8 9) and in the controls 12 1–16 3 (mean 13 9)

The proportion of basophilic erythroblasts within the erythropoietic pool was on average 47% in the EL group and 31% in the controls The difference is significant ($p<0.005$) In the AML+AMML group the corresponding proportion was on average 36% The difference compared to the normals is significant ($p<0.001$) (fig 1)

The proportion of megaloblastoid erythroblasts was on an average 42% in the EL group and 8% in the AML+AMML group The differences are both significant ($p<0.001$) compared to the controls where less than 2% of the erythroblasts were classified as megaloblasts (fig 2)

The average mitotic indices were 54/1,000 in the EL group and 41/1,000 in the AML+AMML group compared to 31/1,000 in the controls The differences are both significant ($p<0.001$) compared to the controls (fig 3)

The number of erythroblastic islands was 15/1,000 erythroblasts in the EL group, 10/1,000 erythroblasts in the AML+AMML group and only 5/1,000 erythroblasts in the controls The differences are both significant ($p<0.001$) (fig 4) compared to the controls

The number of erythroblastic islands was correlated to the percentage of basophilic erythroblasts within the erythropoietic pool $r = 0.54$, $p = 0.001$

Discussion

The present investigation of 34 adult patients with various forms of acute leukaemia was undertaken in order to find out whether a morphologic study of the erythropoietic tissue might give some information on the mechanisms bringing about anaemia in such conditions Since erythropoietic precursor cells are far more accessible in EL than in other forms of acute leukaemia a comparatively large number of EL patients was included in the study The size of the EL group in the present material therefore by far exceeds the real frequency of EL among the myeloid leukaemias

Using ferrokinetic methods, ineffective erythropoiesis has been demonstrated in EL [1] and this is further strengthened by the finding of a maturation arrest in early polychromatic cells in this condition [22] The predominance of basophilic erythroblasts within the erythropoietic pool in EL previously demonstrated by BALDINI *et al* [1] and confirmed in the present work is also consistent with an impaired differentiation towards more mature erythroid precursors Furthermore, a considerable proportion of erythroblasts with a megaloblastoid appearance is known to characterize EL [1, 4, 5, 8, 22] and such changes of the erythroid cells are commonly met with also in other conditions characterized by an ineffective erythropoiesis, e g vitamin B_{12} and folic acid deficiency causing a disturbed DNA synthesis of the erythroid cells [21] Elevated

mitotic indices of the erythroid precursors have also been demonstrated in vitamin B_{12} deficiency and in other disorders where ineffective erythropoiesis may contribute to anaemia [19 20] Our findings of an abnormally large proportion of basophilic erythroblasts the tendency to megaloblastoid morphology and the high mitotic activity are therefore compatible with an ineffective erythropoiesis in EL.

Special interest was devoted to the abundance of erythroblastic islands in the bone marrow of EL patients It has previously been suggested that the presence of such formations in the bone marrow may indicate intramedullary death and phagocytosis of erythroblasts into reticulum cells [3 19 20] thus being a sign of ineffective erythropoiesis The increased numbers of erythroblasts in close contact with or apparently situated within reticulum cells in the EL patients may therefore indicate an intramedullary destruction of large numbers of erythroid precursors in EL

There are several indications that EL and AML are closely related conditions [1 14 18] and that the bone marrow of EL patients as in our cases generally acquires a predominance of myeloblasts after various periods of time It was therefore of interest to note that the abnormalities described above i e the shift to left within the erythropoietic pool the megaloblastic changes the elevated mitotic indices and the increased numbers of erythroblastic islands were also present in the 25 patients with AML or AMML, although the deviations from normal were somewhat less pronounced An increased intramedullary death rate of the erythroblasts may therefore contribute to anaemia in various forms of acute leukaemia It is also probable that an increased destruction of erythroid cells in acute leukaemia is extended to the mature red cells since studies with ^{51}Cr labelled erythrocytes have indicated accelerated damage of such cells in EL [1 5]

The mechanisms causing an increased intramedullary destruction of erythroblasts in acute leukaemic conditions are obscure As mentioned initially there is cytogenetic evidence that the erythroblasts in acute myeloid leukaemia are of leukaemic origin [2 4 11 15 16] and studies with thymidine labelling [4 9 13 17 22] have indicated abnormal kinetic properties It is interesting in this context to note that HUBER *et al* [13] and QUEISSER *et al* [17] found low labelling indices of erythroblasts in AML but that in the present investigation high mitotic indices were found ERNST *et al* [7] found a similar discrepancy between thymidine labelling indices and mitotic indices of erythroblasts in patients with

AML and suggested that the low utilization of labelled thymidine might be a biochemical expression of 'leukaemicness' of the erythroid cells It is thus probable that the erythroid precursors in acute leukaemic conditions are abnormal An increased intramedullary death rate of defective erythroblasts followed by phagocytosis into reticuloendothelial cells would be in good agreement with the results of the present investigation

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Parallel Tubular Structures in Lymphocytes

I Occurrence in Patients with Hodgkin's Disease

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Key Words Hodgkin s disease Lymphocyte inclusions Tubular structures in lymphocytes Ultrastructure of lymphocytes

Abstract Investigations with the electron microscope on peripheral blood of patients with Hodgkin s disease revealed parallel tubular structures in the cytoplasm of the lymphocytes similar to those described in the literature In normal subjects 2 9% of the lymphocytes were found to contain these structures The number of such lymphocytes is increased in most patients as compared to normals A correlation between an increase of inclusions containing lymphocytes and the histological or clinical classifications could not be established An elevation of lymphocytes with tubular structures could be found in both favourable and unfavourable histological and clinical subgroups of Hodgkin patients but especially in all groups with an unfavourable prognosis The origin and function of the parallel tubular structures is still unknown but their increase in lymphocytes of patients with Hodgkin s disease is of some interest in view of the involvement of these cells

The histological features and the impaired cellular immunity have drawn special attention to the lymphocyte in Hodgkin s disease [17, 24] In the peripheral blood the presence of cells of lymphoid origin has been correlated with the activity of the disease [5] These cells are probably from B cell as well as T cell origin [11, 12] Defective function for at least T lymphocytes has been presumed [7 19] Abnormal morphology of the lymphoid cells in the peripheral blood has not been described, but DORFMAN *et al* [6] have found parallel tubular structures in lymphocytes of pathological lymph nodes in Hodgkin s disease Similar structures have been described in lymphocytes of healthy persons by HUHN [14] and were increased in patients with German measles [16] antibody deficiency [15] and rheumatoid arthritis [13] They must be differentiated from the tubu

loreticular (also called tubular) structures, as described a o in patients with SLE, and possibly related to viral infections [8, 9] Although the parallel tubular structures can be found in normal lymphocytes, their increased presence in lymphocytes of some patients with Hodgkin's disease has prompted this investigation to determine in which patients and to what extent these inclusions were present

Materials and Methods

Leukocyte concentrates were prepared from the peripheral blood of 5 healthy persons (laboratory and clinical staff members) and of 24 untreated patients in whom the diagnosis of Hodgkin s disease had been made on the basis of lymph node biopsies. The histological classification was made according to the criteria of the Rye conference on Hodgkin s disease 1965 [20] For clinical staging we used a recently proposed system [10] and the classification proposed at the Ann Arbor conference 1971 [4]

Preparations of peripheral blood cells for electron microscopy Venous blood samples were mixed with EDTA 3% 1 10 and centrifuged at 1 000 rpm for 10 min (Sorvall GLC1) After fixation of the buffy coat with 2% glutaraldehyde in 0 1 m phosphate buffer for 2 h blocks of 1×1 mm were prepared from the cell pellet and rinsed for 24 h in phosphate sucrose buffer Post fixation took place in 2% Palade s OsO_4 for 1 h after which the specimens were embedded in Epon after dehydration with increasing concentrations of alcohol Sections of 0.5 μm were made with a Reichert ultramicrotome for orientation with the light microscope Ultra thin sections were prepared and selected for study with the electron microscope in such a way that cross sections of a certain cell never could be investigated twice Preparations on one hole grids were stained with uranyl acetate and lead citrate From all normal persons and patients, at least in three preparations each 100 lymphocytes were counted with a Philips EM 200 and the percentage of cells containing one or more tubular structures determined Statistical analysis was done with the Student t test for comparison of the mean values of percentages of lymphocytes with parallel tubular structures in each group stud ed

Results

The inclusions found in the cytoplasm of lymphocytes from healthy donors or from patients with Hodgkin's disease did not differ essentially in submicroscopic appearance As described by JORKE [16] and HUHN [14, 15] the most predominant feature is the tubular arrangement in the structures (fig 1) Fully developed tubules measured up to 400 Å in di

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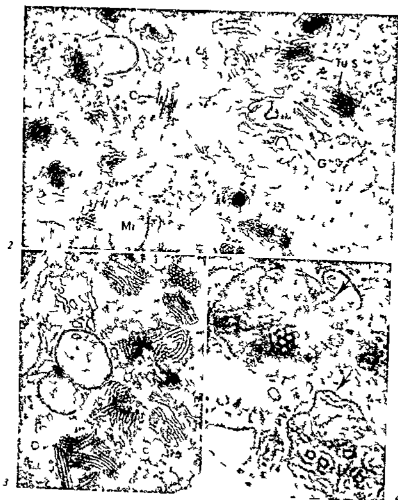
Results

The inclusions found in the cytoplasm of lymphocytes from healthy donors or from patients with Hodgkin s disease did not differ essentially in submicroscopic appearance As described by JORKE [16] and HUHN [14, 15] the most predominant feature is the tubular arrangement in the structures (fig 1) *Fully developed tubules measured up to 400 Å* in di



Fig 1 Lymphocyte of patient with Hodgkin's disease showing tubular structures (Tu S) with partly amorphous material Mi = Mitochondria G Golgi apparatus N — nucleus × 14 600

ameter The tubules are orientated at random within one cell and even within one organelle (fig 3) In a number of the structures amorphous material also can be found Sometimes a thin surrounding membrane can be detected (fig 4) The number of structures per lymphocyte varied in the normal subjects more than 8 inclusions per section were seldom ob



F g 2 Tubular structures (Tu S) local zed around the Golg network (G) A centriole (C) and a primary lysosome are clearly present Mi = M tochondr a × 22 700

F g 3 Deta l of lymphocyte w th tubular structures Note the d fferent d rec t ons of the tubules membranes surround ng parts of the structures are not ev dent × 23 700

F g 4 Deta l of lymphocyte w th inclus ons surrounded by d st nct membranes (arrows) × 50 500

Table 1 Histology, clinical stages and percentage of lymphocytes with parallel tubular structures in 24 patients with Hodgkin's disease

Patient No	Histology	Staging		Tubular structures, %
		Groningen 1972	Ann Arbor	
1	LP	loc	CSIA PSI$_{m\ h\ -}$	10
2	LP	loc	CSIA PSI$_{m\ h\ -}$	14
3	NS	loc	CSIA PSI$_{m\ h\ -}$	5
4	LP	loc	CSIA PSI$_{m\ h\ -}$	23
5	NS	loc	CSIA PSI$_{m\ h\ -}$	20
6	NS	loc	CSIIA PSII$_{m\ h\ -}$	7
7	MT	loc	CSIIA PSII$_{m\ h\ -}$	11
8	NS	loc	CSIIA PSII$_{m\ h\ -}$	12
9	LD	haem diss	CSIIA PSII$_{m\ h-s+}$	32
10	polymorph	haem diss	CSIIA PSII$_{m\ h\ s+}$	34
11	MT/LD	haem diss	CSIIA PSIII$_{m\ h\ s+}$	5
12	LD	haem diss	CSIIA PSIII$_{m\ h-s+}$	10
13	LD	haem diss	CSIIB PSIII$_{m\ h\ s+}$	46
14	MT	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	9
15	LD	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	12
16	MT	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	8
17	LD	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	33
18	LD	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	44
19	LD	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	42
20	LD	haem diss	CSIIIB PSIII$_{m\ h\ s+}$	31
21	LD	haem diss	CSIVB PSIV$_{m\ h\ s+}$	67
22	LD	haem diss	CSIIB PSIV$_{m+h}$	8
23	MT	haem diss	CSIIIB PSIV$_{m+h-s+}$	14
24	NS	haem diss	CSIIBL PSIV$_{m+h}$	23

LP = Lymphocytic predominance, NS = nodular sclerosis, MT = mixed type, LD = lymphocytic depletion

served, whereas in Hodgkin lymphocytes as much as 32 inclusions per section could be found. Usually they were localized around the Golgi apparatus (fig 2), though sometimes they were found more peripherally in the cytoplasm.

The lymphocytes containing the tubular structures all have the same appearance with a chromatin-rich nucleus and a fair amount of cytoplasm. In most cells the Golgi apparatus is well developed for a lymphocyte, the mitochondria are large and round or oval-shaped, the endoplas-

Table II. Statistical analysis (two-sided Student t test) of mean values of lymphocyte percentages with parallel tubular structures in various groups of Hodgkin patients

Group	n	Mean (SEM)	Controls	Localized disease	Stages I+II	LP+NS¹
Controls	5	4.40(1.29)				
Hodgkin patients						
Localized disease	8	12.75(2.17)	$0.01 < p < 0.02$			
Haematogenous disease	16	26.125(4.48)	$0.01 < p < 0.02$	$0.05 < p < 0.10$		
Stages I+II	9	15.11(3.04)	$0.025 < p < 0.05$			
Stages III+IV	15	25.60(4.76)	$0.02 < p < 0.025$		$0.10 < p < 0.20$	
LP+NS	9	13.89(2.23)	$0.01 < p < 0.02$			
MT+LD	15	26.33(4.78)	$0.01 < p < 0.02$			$0.05 < p < 0.10$

¹ See table I for abbreviations.

matic reticulum is scarce, sometimes polyribosomes can be found. Myelin figures can be detected in many lymphocytes.

In the 5 normal persons the percentage of lymphocytes containing one or more of the above described structures varied from 2–9%, mean value 4.40, SEM 1.29. The data on the Hodgkin patients are given in table I, together with the histological and clinical classifications. No correlation could be established between the increased presence of tubular structures and the clinical stage or the histological classification in the individual patient. The data for several subgroups (i.e. the localized and the haematogenous disseminated groups, stages I + II and III + IV, lymphocytic predominance + nodular sclerosis and mixed type + lymphocytic depletion) were compared with each other and with the control group. Statistically significant differences were found in most comparisons (table II). The group of stage III and IV patients merely showed a tendency to be different from stage I + II patients.

Discussion

The observed parallel tubular structures have the same morphology as described in the literature [8, 13, 15, 16, 23]. They should be differentiated properly from other cytoplasmic inclusions such as tubuloreticular

structures [8], which also have been called tubular structures A third type of inclusion has been described by NARDO *et al* [22], but contrary to WHITE [25] in his reference to this observation, we do not consider this to be the same as the parallel tubular structures The ultrastructure of the foregoing inclusions is more like those described in chronic lymphocytic leukemia [1, 2, 21]

Moreover, a simultaneous presence in one cell or transitional forms have not been observed In fact, in our material none of the other cytoplasmic abnormalities in lymphocytes have been found so far Contrary to the findings in SLE [8] we think that the increased presence of the parallel tubular structures per cell and of the percentage lymphocytes containing them may have significance in Hodgkin's disease Their association with clinically or histologically unfavourable groups suggests a connection with the progression of the disease, although this does not hold true for each individual patient Other features of Hodgkin's disease as the impairment of cellular immunity [19] or the elevation of viral antibodies [18] also parallel the progression of the disease when studied in groups of Hodgkin patients

The significance of the occurrence of the lymphocytes with parallel tubular structures is difficult to interpret, because it largely depends on the origin and the function of these structures A connection with mitochondria or centrioles has been mentioned, but a special relationship or transitions with these organelles have not been observed in our material A function as cytosomes, put forward by HUHN [15] or more specially as segresomes concerned with segregation of small molecular substances reaching the interior of the cell by transmembraneous transport [16] is not clear The tubular arrangement is at variance with the normally granular structure of cytosomes Also, the increase of the inclusions as now observed in Hodgkin's disease must then indicate a strongly raised lysosomal activity in lymphocytes

A lipoprotein nature of the tubules is proposed [16] This could point to digestive processes or degradation of (own) cell constituents It would be in accordance with the opinion of REYES *et al* [23], that they are degenerative cell organelles such as mitochondria after *in vitro* manipulation Similar parallel layers (myelin forms) can be seen in the digestion of erythrocytes by macrophages [3] In Hodgkin's disease the lymphocytes must then have an increased vulnerability

Whatever the origin or function of the parallel tubular structures may be, their increased incidence in patients with Hodgkin's disease probably

represents lymphocytic changes due to alterations in the cell constituents or the cell functions Especially in this disease, where the role of the lymphocyte attracts so much attention, we consider the observed abnormalities deserve further investigation

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Defect of Bone Marrow Granulocyte Reserve in Viral Hepatitis

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Key Words Aplastic anemia Bone marrow Etiocholanolone Granulocyte reserve Viral hepatitis

Abstract In patients with HBAg positive and HBAg negative viral hepatitis the etiocholanolone test showed a decrease of the bone marrow granulocyte reserve at the height and, though at a lower degree at the end of the disease These results are discussed in relation with the incidence of aplastic anemia following viral hepatitis

In recent years several studies reported the occurrence of bone marrow failure following viral hepatitis [1–15] The pathogenesis of this complication still deserves elucidation Furthermore, it is not yet clear whether in any case of viral hepatitis minor alterations of hemopoiesis are being produced which might generally escape the current routine hematological investigations

With reference to granulocytopoiesis, a substantially normal leukocyte count may coexist with altered relations between the size of the large intramedullary granulocyte storage pool and that of the marginal and of the circulating granulocyte pools [16, 17] Several authors [18–22] have shown that the marrow granulocyte reserve can be assayed by studying the peripheral leukocytic response to injection of pyrogens In the studies reported no purified endotoxin but a safer and more effective agent, etiocholanolone [23], was employed In this paper we shall present the results of the etiocholanolone test on marrow granulocyte reserve in viral hepatitis

Materials and Methods

Patients A total of 26 patients 20 males 6 females aged 15–72 years were studied 16 suffered from HBAg positive viral hepatitis and 10 from HBAg negative hepatitis

The diagnosis of viral hepatitis has been based on anamnestic epidemiological clinical and laboratory criteria the HBAg positive cases being classifiable as B hepatitis, whereas the HBAg negative cases were interpreted as instances of A hepatitis according to the criteria mentioned above In 8 patients the studies were performed within the first 13–20 days since the presumed date of the beginning of the illness However as aplastic anemia is generally a late complication of hepatitis the remaining 18 patients were investigated at the end of the illness immediately before dismission from the Institute

Care was taken to exclude from this study all patients bearing conditions capable of modifying the granulocyte response (bacterial infections concomitant treatment with corticoids etc) A group of 7 healthy controls 5 males and 2 females aged 20–58 was also studied after careful screening of their clinical conditions Both the patients and the controls were fully informed before giving their consensus to these studies

Etiocholanolone test The method of KIMBALL *et al* [23] was followed Exactly at midnight each individual received an intramuscular injection of 0 2 mg kg of a 1‰ (w/v) solution of etiocholanolone in propylene glycol (Vister Casatenovo Brianza Italy) Immediately before and 9 12 and 15 h after the injection triplicate peripheral leucocyte counts were performed by an automatic device and the leukocyte formula was determined by counting at least 200 cells in smears stained according to the May Grünwald Giemsa method The absolute number of granulocytes/µl was finally calculated The times of blood sampling had been chosen in order to minimize variations due to circadian rhythm of granulocyte production and of cortisol production the latter hormone being able to modify the granulocyte response to etiocholanolone Pyrogen free needles and syringes were employed Furthermore the patients had to be rigorously apyretic in the 3 days preceding these studies in order to be included in this study The height of the leukocyte response was evaluated in relation to the granulocytic increment (ΔG) that is the difference between the preinjection value and the maximum absolute granulocyte count obtained at any one of the hours following the injection of etiocholanolone ΔG was considered normal when the increase in granulocytes was higher than 2 600 µl after the injection of etiocholanolone

Results

The results have been summarized in table I As it may be noted there was a moderate number of pathological values among all the groups considered but if we disregard positivity or negativity of HBAg in the blood we find a total of 3 pathological ΔG over 8 at the height of the disease and 1 over 18 at the dismission from the hospital On the contrary no pathological ΔG were found in the controls Furthermore the average ΔG of the patients at the height of the disease and of those at the moment of recovery were lower than the ΔG of the controls If the variance analysis was applied to these data the differences were statistically significant

Table I The etiocholanolone test in viral hepatitis and in healthy controls (values expressed as numbers of granulocytes/µl ± SE)

	Number of observations	Baseline	9th h	12th h	15th h	IG
Controls	10	6 650	12 115	14 230	11 742	8 675
		± 502	± 1 249	± 1 057	± 971	± 1 856
Acute phase						
HBAg-negative	3	2 641	4 950	6 389	6 423	4 265
		± 443	± 508	± 680	± 1 470	± 1 520
HBAg-positive	5	3 073	4 693	5 835	5 565	2 970
		± 318	± 510	± 972	± 750	± 958
Average	8	2 924	4 789	6 075	5 887	3 456
		± 273	± 351	± 625	± 665	± 793
Remission						
HBAg-negative	7	3 350	7 312	8 636	7 418	5 571
		± 216	± 800	± 1 255	± 1 343	± 855
HBAg-positive	11	3 125	5 945	7 557	6 941	5 302
		± 250	± 744	± 697	± 876	± 761
Average	18	3 224	6 478	7 996	7 126	5 407
		± 183	± 578	± 639	± 725	± 562
Overall average	26	3 132	5 958	7 405	6 745	4 807
		± 155	± 438	± 508	± 547	± 455

($p<0.05$). As far as differences between HBAg− and HBAg+ patients are concerned, the mean IG of HBAg+ patients was lower than that of HBAg− patients, though these differences might probably be irrelevant on statistical grounds, due to numerical imbalance between groups.

Discussion

The etiocholanolone test showed that in a small, though appreciable number of hepatitis patients, the granulocyte response is impaired and that even if normal values are comprised in the average values, the overall granulocyte response is significantly lower than in the controls. This decrease was observed at both the height of the disease and – though at a lower degree – on recovery from the disease, possibly with a relatively more severe compromission of the granulocyte reserve in HBAg+ patients compared with HBAg− patients. Though this reduction in marrow reserve was not obligatorily accompanied by a basal neutropenia, the re-

sults mean, however, an impairment of the granulocyte response in respect to different stimuli, the same response in larger part than normal being confided to exchanges between the marginated and the circulating pool of granulocytes The mechanisms conditioning such a defect are not easy to explain, though they might result from a derangement of cellular mechanisms in earlier phases of the life cycle of granulocytes, such as differentiation proliferation and maturation of bone marrow granulocyte precursors It would seem that this derangement lies in myelotoxic immunoallergic, or other causes, as each one of the theories proposed in order to explain the damage of hemopoiesis induced by virus [1, 4, 7, 11, 24–28] would be suitable for interpretation of our results At the height of the disease such subtle alterations of granulocytopoiesis (and possibly of the production of erythrocytes and thrombocytes) may be present, which in rare instances could further progress until the development of cytopenic syndromes associated with, or following hepatitis [2 4, 6–8, 10, 11, 25, 29, 30]

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Detection of Carriers of Haemophilia A

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Key Words Carriers of haemophilia Factor VIII related antigen Haemophilia

Abstract In a series of 18 known and 14 possible carriers of haemophilia A the factor VIII related antigen was assayed Comparison of results with the biological activity of factor VIII detected correctly 17 of 18 known carriers On the other hand the carrier state was detected in 8 of 14 possible carriers The results are similar to those of other investigators

Until recently, the detection of carriers of haemophilia A depended upon the measurement of biological activity of factor VIII By that method about half of the known carriers were detected as normal [5] The development of an immunoassay for factor VIII [7] demonstrated that the plasma of haemophilic patients contains normal amounts of a protein with similar antigenicity to factor VIII The plasma of normal persons contains this protein in an approximate proportion 1 1 to the biological activity of factor VIII, while in the plasma of known carriers the ratio of activity to antigen was found lower than in normal persons, up to 1 2 [6] By comparison of the biological activity of factor VIII with factor VIII-related antigen, 90–95% of known carriers are correctly detected This means that the method is reliable for detecting the carrier state amongst possible carriers [1, 2, 4, 6]

We examined in our laboratory 18 known carriers by this method and 17 of them were correctly detected Application of this method to a group of 14 possible carriers detected the carrier state in 8 of these

Materials and Methods

Assay of biological activity of factor VIII and factor VIII related antigen was carried out in the plasma of 23 normal women (not in pregnancy or oral contracep

tive therapy) aged 20–40 years, 18 known carriers of haemophilia A, and 14 possible carriers

As carriers were considered the mothers of two haemophilic sons or the mothers of one haemophilic son but with a family history of haemophilia, or the daughters of haemophiliac patients As possible carriers were considered the daughters of known carriers

The biological activity of factor VIII was assayed by *the one stage method*

The factor VIII related antigen was assayed by quantitative immunoelectrophoresis [3] in 1% agarose containing 4‰ of antiserum to factor VIII related antigen (Behring) As a standard, pooled normal plasma prepared from the plasma of 15 normal men aged 25–40 years, was used Each plasma was tested at two dilutions and the results were averaged In 1 ml of pooled normal plasma a unit of biological activity of factor VIII and a unit of factor VIII related antigen were present

The regression line of 23 normal women was calculated by the method of least squares and the 95 and 99% confidence belts by the statistical method

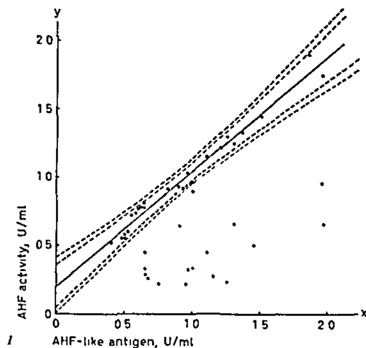
Results and Discussion

As can be seen from table I and figures 1 and 2, the measurement of functional factor VIII titre on 18 known Greek haemophilia carriers detects the carrier state in 11 of these, while comparison of biological activity of factor VIII to factor VIII related antigen showed diagnostic differences from normal in 17 (fig 1), a finding similar to that of other investi-

Table I Comparison of results of 23 normal women 18 known carriers and 14 possible carriers of haemophilia A

Cases	Range of ratio A/Ag[1]	Arithmetic ratio of activity U/ml	Arithmetic ratio of antigen U/ml	Mean ratio A/Ag
23 normal women	0 89 1 30	0 98	0 92	1 06
18 carriers of haemophilia A	0 19–0 96	0 48	1 07	0 44
8 possible carriers found as carriers	0 26–0 88	0 47	0 68	0 69
6 possible carriers found as normal	0 93–1 26	1 05	0 96	1 09

[1] A = Activity Ag = antigen



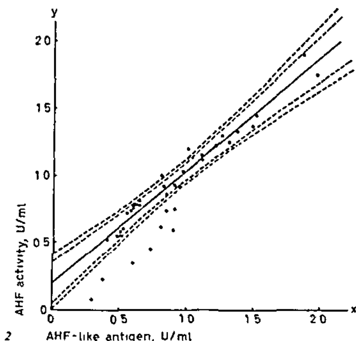


Fig 1 Relation of AHF activity to AHF-related antigen in normal women (●) and in carriers of haemophilia (+) The regression line and the 95 and 99% confidence belts of normal women are indicated $y = 0\ 204 + 0\ 845\ x$

Fig 2 Relation of AHF activity to AHF-related antigen in normal women (●) and in possible carriers of haemophilia (+) The regression line and the 95 and 99% confidence belts of normal women are indicated $y = 0\ 204 + 0\ 845\ x$

gators Application of the method to 14 possible carriers identified the carrier state in 8, while the determination of functional factor VIII activity alone detected the carrier state only in 4 of these (fig 2) As the 14 possible carriers are young girls it is impossible at the moment to assess the reliability of the method

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Human Platelet Aggregation by Mixed Cryoglobulins

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Key Words Cryoglobulinemia Glomerulonephritis Immune complexes Platelet aggregation

Abstract Glomerulonephritis in idiopathic mixed cryoglobulinemia represents perhaps a glomerular damage by immune complexes

In this study a sigmoidal like curve was obtained after addition of 13 different mixed cryoglobulins to both autologous and isologous platelet rich plasma tested in platelet aggregometer The lag phase of the curve corresponds to platelet phagocytosis of cryoglobulin binding ferritin as shown in electron microscopy and the optical density decrease phase corresponds to the aggregation of platelets that shows the same ultrastructural characteristics of ADP induced platelet aggregation This platelet aggregation is inhibited by different drugs Intraglomerular platelet aggregation by cryoglobulins might play a key role in determining the glomerular damage in cryoglobulinemia by the release of nucleotides and vasoactive amines

Lerner and Watson [12] in 1947 named cryoglobulins some human serum proteins which precipitate reversibly at 4 °C Cryoglobulins may represent both a secondary phenomenon in monoclonal gammopathies [22, 26], viral and bacterial diseases [11, 27] systemic disorders [24] *and a primary phenomenon in a syndrome called idiopathic mixed cryo* globulinemia (IMC) [16] Most cases of IMC develop glomerulonephritis which represents, perhaps, glomerular damage by immune complexes [5 6 13 14 26] Ultrastructural findings showed platelet aggregates in glomerular vessels [13], moreover, various experimental studies report platelet aggregation by immune complexes [10, 17, 18]

These data suggest that platelet aggregation may play a key role in glomerular damage during cryoglobulinemia The purpose of this work is to study the action of cryoglobulins on human platelet aggregation in various experimental conditions

Material and Methods

Reagents Acetylsalicylic acid (ASA Bayer) 1 mg/ml DB-cAMP (kindly provided by Carlo Erba Research Laboratories) 10 mg/ml and adenosine (Sigma) 1 mg/ml were stored in a stock solution at 20 °C and diluted in saline just before the assay to the final concentration desired

Prostaglandin E1 (PGE1 Upjohn 6) was stored in a stock alcoholic solution of 1 mg/ml at -20 °C and diluted in saline just before the assay

Stock solutions of both heparin (Liquemin Roche) diluted in saline to 10 IU/ml and thrombin (Topostasine Roche) dissolved in saline to 30 NIH units ml were kept at -20 °C in small aliquots until use

Isolation of heparinized platelet rich plasma (PRP) and platelet poor plasma (PPP) Blood samples from healthy not recently treated subjects were added to heparin (2 U/ml) Blood was centrifuged at 400 *g* for 10 min to obtain PRP and at 2 000 *g* for 20 min to obtain PPP Platelet concentration in the normal PRP samples ranged between 250 000 and 500 000 mm³ All experiments were performed during the 3 h following collection of the blood All equipment for handling blood and plasma was plastic

Gel filtered platelets (GFP) were obtained by the method of TANGEN et al [25] A specimen of GFP incubated for 1 h with fluorescein labelled antisera against human C_3 showed no complementary fraction by immunofluorescence microscopy

Isolation of cryoglobulins Cryoglobulins (10 IgM IgG 2 IgA IgM IgG 1 IgG IgG) were obtained from 13 patients affected by IMC whose cryocrit, obtained by the method described by BALESTRIERI et al [1] ranged between 10 and 15% The method described by MELTZER and FRANKLIN [15] was followed for isolation of cryoglobulins Immunoelectrophoresis analysis of washed cryoglobulins challenged with anti β_1C serum did not show any precipitation line

Ferritin labelled cryoglobulin 50 mg of carbodiimide (1-ethyl 3[3-dimethyl aminopropyl]) carbodiimide hydrochloride (Ott Chemical Company, Myskegon Mich) and 10 mg of ferritin (cadmium free 2 cryst Fluka AG 46230) suspended in 1 ml of PBS were added to 100 mg of the IgG IgG cryoglobulin This suspension was kept at 40 °C for 30 min and then at 4 °C for 2 h After centrifugation at 10 000 *g* at 4 C for 20 min the pellet was resuspended and the solution was dialyzed in 5 000 ml of PBS at 4 °C

Platelet aggregometry The changes in optical density of PRP were measured in an EEL 169 platelet aggregation meter (Evans Electroselenium Ltd) connected to a pen recorder (Servogor Linear/Logarithmic Integrating Recorder) to permit the automatic registration of variations in transmitted light Sensitivity was adjusted so that PPP or buffer set close to 100% and PRP or GFP to 0% Reagents were added to the cuvette of the aggregometer as follows 0 8 ml PRP or GFP 0.2 ml buffer or various reagents and 0 2 ml cryoglobulin Platelet aggregometry was performed with the stirrer on

Biological titrations Platelet factor 3 (PF_3) availability (Stypven time) was determined according to SPAET and CINTRON [23] Simultaneous determination of platelet aggregation and PF_3 availability was obtained as described by HARDISTY and HUTTON [7]

Platelet factor 4 (PF_4) release was measured directly in the aggregometer as heparin thrombin clotting time (HTT) 0 8 ml citrated PRP was tested with 0 2 ml cryoglobulin, at various times from the beginning of the reaction standardized concentrations of both heparin (1 5 IU/ml, 0 2 ml) and thrombin (10 NIH units/ml, 0 4 ml) were rapidly added to the mixture The phirst filaments of fibrin were detected as a sudden increase in optical density and heparin thrombin clotting time was then calculated

Electron microscopy Isologous platelets tested against ferritin labelled cryoglobulin were transferred at different times of the sigmoidal curve from the aggregometer cuvette to a 2% glutaraldehyde solution buffered in phosphate After 30 min platelets were centrifuged and the pellet postfixed in OSO_4 dehydrated and embedded in Epon Araldite mixture according to routine procedure As a control platelets of the same donor were fixed after 10 min stirring with a suspension of noncoupled ferritin that did not produce a decrease in optical density in the aggregometer Similarly, samples of PRP and GFP incubated with noncoupled cryoglobulin were fixed for electron microscopy Ultrathin sections obtained with an LKB ultratome were double stained with uranyl acetate and lead citrate and observed under a Hitachi HU 11A electron microscope

Results

Platelet Aggregation by Cryoglobulins in PRP

Figure 1 shows the typical aggregation curve observed when a suspension of cryoglobulin was added to both autologous and isologous PRP The curve has a sigmoidal collagen-like pattern with a period of upper plateau corresponding to the lag phase and a period of fast decrease of optical density, corresponding to a sudden appearance of macroscopic platelet clumps This phenomenon was not reversible and a second aggregation wave was never observed The lag phase was never shorter than 3 min and increased progressively reducing the concentration of cryoglobulins Moreover, there was a direct relationship between the decrease of optical density and the initial number of platelets A marked increase of PF_3 availability was observed just at the end of the lag phase, while the release of PF_4 was a phenomenon contemporaneous and proportional to the aggregation as shown in the curve (fig 1)

Effect of Anticoagulants and Platelet Aggregation Inhibitors

Platelet aggregation by cryoglobulins was optimal when heparin at low final concentration (2 IU/ml) was used as anticoagulant Higher doses of heparin (15 IU/ml) and sodium citrate (6 mg/ml) increased the lag phase and reduced the degree of platelet aggregation (table I) In presence of

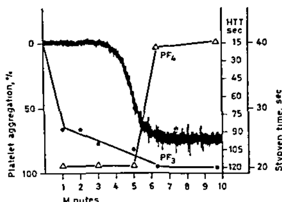


Fig 1 Platelet aggregation by cryoglobulin correlated with PF_3 availability (Stypven time, ●) and PF_4 release (HTT, △)

Table I Effect of anticoagulants on platelet aggregation by cryoglobulin

	Lag phase min	Aggregation %
Heparin, 2 U/ml	5	80
Heparin, 15 U/ml	6	50
Trisodium citrate, 6 mg/ml	8	30
EDTA, 5×10^{-3} M	30	–

EDTA (5×10^{-3} M) platelet aggregation was completely inhibited Platelet aggregation by cryoglobulins was also partially or totally inhibited by several inhibitors of aggregation by ADP, such as adenosine (6×10^{-4} M), DB-cAMP (3×10^{-3} M), PGE1 (9×10^{-5} M) Aggregation was also inhibited by ASA (4×10^{-4} M), a platelet release reaction inhibitor

GFP Aggregation by Cryoglobulins

Figure 2 shows the curve obtained by adding the cryoglobulin to a sample of GFP stirred in the aggregometer at 37 °C The aggregation curve is similar to that observed with PRP, except that aggregation started more promptly in the absence of plasma Similarly the reaction was totally inhibited by EDTA (5×10^{-3} M), adenosine (6×10^{-4} M) and ASA (4×10^{-4} M)

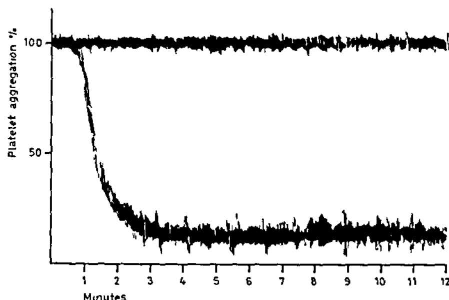


Fig 2 Aggregation of gel filtered platelets induced by cryoglobulins in buffer (lower curve) Inhibitory effect of various substances 5×10^{-3} M EDTA 6×10^{-4} M adenosine, 4×10^{-4} M acetylsalicylic acid (upper curve)

Ultrastructural Findings

After 2 min, during the lag phase of the curve, platelets incubated with ferritin-labelled cryoglobulin are separated and the majority shows rounded bodies, single or multiple, with a dark matrix (fig 3–5) Moreover, they are more electron dense than the regular dense granules of control samples incubated with nonlabelled cryoglobulin These rounded bodies are centrally or peripherically located and frequently in close relation with the membranous system which outlines vacuoles or invaginations of the cytoplasmic membrane In nonstained ultrathin sections, they maintain their electron density (fig 5b) similar to that of ferritin particles present in the extracellular space These findings support the hypothesis that the rounded bodies might contain ferritin molecules taken up by the platelets together with the cryoglobulin At the time corresponding to the end of the sigmoidal curve, platelets incubated with labelled cryoglobulin are clumped in aggregates ultrastructurally similar to those induced by ADP (fig 4)

In the control samples incubated with noncoupled ferritin or unlabelled cryoglobulin, platelets do not show the rounded dense bodies pre-



Fig 3 Isologous platelets incubated with ferritin labelled cryoglobulin at 2 min after stirring in the aggregometer Platelets show rounded bodies (arrows) which are more electron dense than the regular granules × 12 000

Fig 4 Isologous platelets incubated with labelled cryoglobul n at the end of the s gmoidal curve Platelets are clumped in aggregates which present some res dual regular dense granules and peripheral pseudopods × 7,500

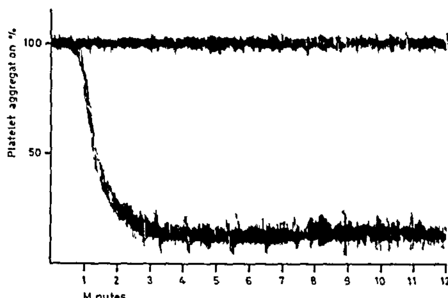


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Fig 3 Isologous platelets incubated with ferritin labelled cryoglobulin at 2 min after stirring in the aggregometer Platelets show rounded bodies (arrows) which are more electron dense than the regular granules × 12 000

Fig 4 Isologous platelets incubated with labelled cryoglobulin at the end of the sigmoidal curve Platelets are clumped in aggregates which present some residual regular dense granules and peripheral pseudopods × 7,500

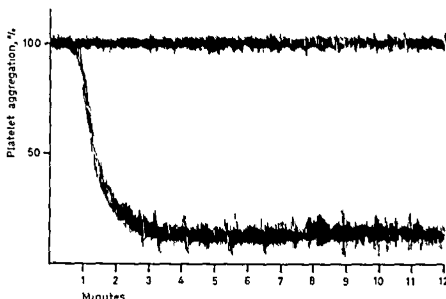


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F g 3 Isologous platelets ncubated with ferr t n labelled cryoglobul n at 2 m n after st rr ng n the aggregometer Platelets show rounded bod es (arrows) wh ch are more electron dense than the regular granules × 12,000

F g 4 Isologous platelets ncubated with labelled cryoglobul n at the end of the s gmo dal curve Platelets are clumped n aggregates wh ch present some res dual regular dense granules and peripheral pseudopods × 7,500



Fig 5 Isologous platelets incubated with ferritin labelled cryoglobulin at 2 min of the sigmoidal curve A rounded body (arrow) with a very electron dense matrix is evident in the platelet cytoplasm In nonstained sections (b) these bodies maintain their electron density similar to that of ferritin particles present in the extracellular space × 17 000

viously described Ferritin particles are present in the extracellular space and do not enter into cells whereas unlabelled cryoglobulin causes platelet aggregation Platelet aggregates were also observed after addition of unlabelled cryoglobulins in GFP samples

Discussion

The decrease of optical density obtained in the aggregometer by the action of human cryoglobulins on PRP depends on the formation of platelet aggregates similar to those observed with ADP Moreover, the inhibition of platelet aggregation by cryoglobulin in the presence of DB cAMP, adenosine and PGE1 emphasizes the key role of ADP in this reaction It is known from experimental works on laboratory animals that

immune precipitates are phagocytized by platelets and this phenomenon is associated with amines and nucleotides release [4–17] Our ultrastructural data suggest that platelets might phagocytize the cryoglobulin during the lag phase of the aggregation curve at which time there is an optimal availability of PF_3 The inhibition of platelet aggregation by ASA supports the hypothesis that cryoglobulin phagocytosis by platelets is followed by release reaction and platelet aggregation [28] At this point the question may be raised whether aggregation by cryoglobulins may play a role in the pathogenesis of clinical vasculitis observed in IMC and particularly in the pathogenesis of the proliferative glomerulonephritis IgG and IgM immunoglobulin deposits were observed by immunofluorescence in the glomerular endothelium in patients affected by IgM IgG idiopathic cryoglobulinemia [6, 16 26]

Glomerulonephritis associated with IMC appears to be a model of glomerular damage by immune complexes It is likely that several factors such as complement, neutrophils and platelets take part in the pathogenesis of small vessel wall damage induced by immunocomplexes [2, 8, 14] Experimental studies on laboratory animals have shown that platelets are involved in this process by several mechanisms, some of which are mediated by complement and some not Vasoactive amines, responsible for glomerular damage, should be released from such activated platelets [3 8, 21] Our results in man suggest that platelets may play a very important role in glomerular damage by cryoglobulin In fact, cryoglobulins induce the release of vasoactive amines by platelets during the aggregation process

Many experiments done on the reaction of immune complexes on platelets have shown a striking difference between washed platelets and PRP, the first ones showing an immediate release and aggregation, whereas in plasma almost exclusively a late reaction is observed [9, 19, 20] This agrees with our observation that GFP show a more prompt reaction with cryoglobulin

Moreover the action of cryoglobulins on human platelets do not depend necessarily on the presence of complement In fact platelet aggregation is also observed when we tested washed cryoglobulins on gel filtered platelets both of which are free of β_1C globulin Such a role of this nature for platelets may open some interesting therapeutical prospect Drugs inhibiting platelet aggregation may be combined with anticoagulants to prevent both inflammatory injury and intraglomerular coagulation In one patient (IgG IgG cryoglobulinemia) treatment with ASA and heparin

was successful This treatment induced a spectacular regression of the acute renal failure due to severe proliferative glomerulonephritis Fibrinoid thrombi were present in glomerula loops before therapy

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Influence of Cytotoxic Drugs on Platelet Functions *in vitro*

III Peptichemio*

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Key Words Cytotoxic drugs Peptichemio® Platelet functions Tumour chemotherapy

Abstract The influence of Peptichemio® (m dichloroethyl amino-l phenyl alanyl peptide complex) on the following platelet functions was studied aggregation release of platelet factor 4, availability of platelet factor 3, acid phosphatases and reptilase clot retraction Concentrations of the drug corresponding to that found in plasma of treated patients did not significantly influence platelet functions Concentrations 10 times higher brought about an inhibition It is concluded that after current dosage of Peptichemio, bleeding disorders caused by impairement of platelet functions are improbable

Peptichemio® is the trade name for a m-dichloroethyl-amino l phenylalanyl-peptide complex with antitumour activity, which was recently prepared by DE BARBIERI *et al* [1, 2] The complex has the property of both alkylating and antimetabolic substances and it was successfully used in the treatment of different haemoblastoses, malignant lymphomas and/or solid tumours [4, 5, 10] Similarly to the other cytotoxic drugs, Peptichemio can produce, particularly in higher doses, bone marrow hypoplasia or aplasia with consequent leukopenia and/or thrombocytopenia The latter is often associated with haemorrhagic manifestations However, defects in haemostatic platelet plug formation caused by impairment of platelet functions could also play a part in pathogenesis of bleeding disorders after the administration of cytotoxic drugs That is why we investigated the influence of these compounds on some platelet functions [8, 9] In the present paper we studied the effects of Peptichemio on

[1] The authors wish to thank Dr B CHAMBRETTE (Stago) and Dr K STOCKER (Pentapharm) for kind supply of reptilase and collagen

platelet aggregation on release of platelet factor 4 (PF4) availability of platelet factor 3 (PF3) availability of acid phosphatases (APh) and on reptilase clot retraction (RCR)

Material and Methods

Blood was taken from healthy volunteers, mixing 9 parts of venous blood with one part of 3.8% trisodium citrate. Platelet rich plasma (PRP) was obtained by centrifuging the blood at 280 g for 20 min at 16 °C. The platelets were counted according to the method of PILTTE and PIETTE [11] and adjusted to 300 000 (±10 000) µl with platelet poor plasma (PPP) from the same donor. PPP was prepared similarly by centrifuging the blood at 12,000 g for 20 min at 12 °C. Collagen (Stago) ADP (Sigma) and Adrenalin (Spofa) were used and diluted to the required concentration by adding Michaelis buffer at pH 7.35 approximately. Peptichemio (Istituto Sieroterapico Milanese Serafino Belfanti) was dissolved to the required concentration in 5% glucose. PF3 and APh availability, release of PF4 and RCR were tested according to the methods described elsewhere [6, 7]. Aggregation was estimated according to the Born's method, using modification according to SINACOS and CAEN [12].

Influence of Peptichemio on platelet functions was tested using different concentrations of the drug, i.e. 15, 30 and 150 µg/ml of PRP following incubation of 5 and 30 min in water bath at 37 °C. The lowest concentration corresponds approximately to that supposed in plasma following administration of therapeutic dose in man.

Results

Concentrations of 15 and 30 µg of Peptichemio did not produce significant alterations in platelet aggregation and RCR regardless of the inducer used and the duration of incubation (fig. 1–3, table IV). The concentration of 150 µg of Peptichemio/ml of PRP significantly inhibited platelet functions. Following this high dose of Peptichemio similar striking or even complete inhibition of release of PF4 and significant decrease of PF3 and APh availability were found (tables I–III). However availability of APh and particularly availability of PF3 and release of PF4 were partly influenced also by lower concentrations of Peptichemio and that especially following induction with ADP and adrenalin (tables I–III). Duration of incubation also plays a role here, as after incubation of 30 min release of PF4 was found to be significantly inhibited even with a dose of 15 µg/ml using ADP as inductor. As far as availability of APh and PF3 is concerned the mentioned decrease was preceded by an increase instantaneously after addition of Peptichemio (tables II, III).

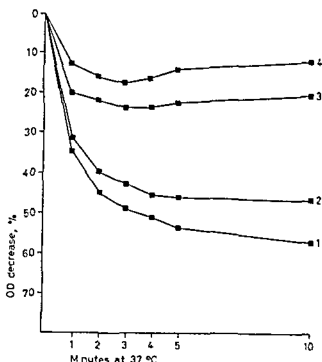


Fig 1 Influence of Peptichemio on platelet aggregation induced by ADP (4.5×10^{-6} M) at 37 °C 1 = PRP + Michaelis buffer, 5–30 min + ADP, 2 = PRP + Peptichemio 30 μg/ml, 30 min + ADP, 3 = PRP + Peptichemio 150 μg/ml, 5 min + ADP, 4 = PRP + Peptichemio 150 μg/ml 30 min + ADP

Table I Influence of Peptichemio on release of PF4 after induction with ADP (4.5×10^{-6} M), adrenaline (6×10^{-6} M) and collagen (20 μg/ml) the values indicate the quantity of PF4 (% + SD) released in the 10th min following addition of the inducer at 37 °C

PRP +	ADP	Adrenaline	Collagen
Michaelis buffer (pH 7 35), 0–60 min	46 2 ± 2 4	46 6 ± 1 5	52 2 ± 1 7
Peptichemio 15 μg/ml, 5 min	15 1 ± 2 5	37 6 ± 3 8	40 4 ± 1 3
Peptichemio 15 μg/ml, 30 min	3 4 ± 3 1	20 8 ± 9 1	38 8 ± 1 9
Peptichemio 30 μg/ml, 5 min	14 5 ± 6 6	25 7 ± 9 6	30 5 ± 6 1
Peptichemio 30 μg/ml, 30 min	3 0 ± 2 8	9 5 ± 1 8	30 8 ± 6 1
Peptichemio 150 μg/ml, 5 min	0 8 ± 0 7	2 4 ± 1 9	6 5 ± 2 0
Peptichemio 150 μg/ml, 30 min	0 5 ± 0 3	1 2 ± 1 0	2 2 ± 1 8

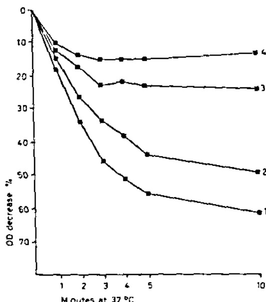


Fig 2 Influence of Peptichemio on platelet aggregation induced by adrenaline (6×10⁻⁶ M) at 37 °C 1 = PRP + Michaelis buffer, 5–30 min + adrenaline, 2 = PRP + Peptichemio 30 μg/ml, 30 min + adrenaline, 3 = PRP + Peptichemio 150 μg/ml 5 min + adrenaline, 4 = PRP + Peptichemio 150 μg/ml, 30 min + adrenaline

Table II Influence of Peptichemio on PF3 disponibility after induction with ADP (4 5 × 10⁻⁶ M), adrenaline (6 × 10⁻⁶ M) and collagen (20 μg ml), the values indicate the quantity of PF3 (% + SD) disponible in the 20th min following addition of the inducer at 37 °C

PRP +	Buffer	ADP	Adrenaline	Collagen
Michaelis buffer (pH 7 35), 0–60 min	0 2±0 1	30 0±3 6	32 4±2 1	31 2±3 8
Peptichemio 15 μg ml, 5 min	1 0±0 3	27 7±7 5	27 2±8 1	29 3±4 3
Peptichemio 15 μg ml, 30 min	1 8±0 4	10 3±3 5	15 4±6 6	22 2±8 2
Peptichemio 30 μg/ml, 5 min	2 1±0 5	9 8±1 4	20 1±5 6	26 2±4 3
Peptichemio 30 μg/ml, 30 min	4 2±1 2	8 0±2 7	11 8±3 2	18 3±3 1
Peptichemio 150 μg/ml, 5 min	3 9±0 7	9 5±3 6	9 4±4 1	12 1±2 5
Peptichemio 150 μg/ml, 30 min	5 5±1 8	5 4±0 7	5 9±1 1	8 5±3 3

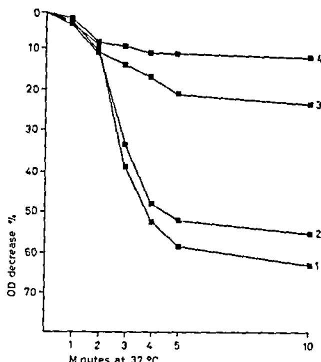


Fig 3 Influence of Peptichemio on platelet aggregation induced by collagen (20 μg/ml) at 37 °C 1 = PRP + Michaelis buffer, 5-30 min + collagen, 2 = PRP + Peptichemio 30 μg/ml, 30 min + collagen, 3 = PRP + Peptichemio 150 μg/ml, 5 min + collagen, 4 = PRP + Peptichemio 150 μg/ml, 30 min

Discussion

The results demonstrated that, *in vitro*, therapeutic concentrations of Peptichemio did not significantly influence platelet functions Release of PF4 was, however, inhibited even with the concentration of 15 μg of Peptichemio, but that was significant only after induction with ADP, following 30-min pre-incubation After shorter lasting pre-incubation, the inhibition was only of a moderate degree Although *in vivo* conditions could differ from those *in vitro*, significant changes in platelet functions in treated patients can hardly be expected, especially as far as in actual platelet population However, the question remained, whether Peptichemio, which is capable to produce bone marrow hypoplasia or aplasia [1, 4], could not change metabolism and functions of megakaryocytes This new platelet population would have to be studied separately

Table III Influence of Peptichemio on APh disponibility after induction with ADP (4 5 × 10^{-4} M), adrenaline (6 × 10^{-4} M) and collagen (20 µg/ml), the values indicate the quantity of APh (µMol ml/h ± SD) disponible in the 20th min following addition of the inducer at 37 °C

PRP +	Buffer	ADP	Adrenaline	Collagen
Michaelis buffer (pH 7 35), 0–30 min	0 010 ± 0 005	0 822 ± 0 137	0 982 ± 0 089	0 977 ± 0 075
Peptichemio 15 µg/ml, 5 min	0 058 ± 0 028	0 824 ± 0 148	0 815 ± 0 114	0 808 ± 0 114
Peptichemio 15 µg/ml 30 min	0 088 ± 0 030	0 473 ± 0 118	0 737 ± 0 167	0 735 ± 0 167
Peptichemio 30 µg/ml, 5 min	0 150 ± 0 038	0 539 ± 0 100	0 754 ± 0 197	0 745 ± 0 194
Peptichemio 30 µg/ml, 30 min	0 250 ± 0 050	0 361 ± 0 063	0 633 ± 0 205	0 702 ± 0 225
Peptichemio 150 µg ml, 5 min	0 205 ± 0 050	0 399 ± 0 130	0 353 ± 0 148	0 344 ± 0 148
Peptichemio 150 µg/ml, 30 min	0 485 ± 0 064	0 482 ± 0 031	0 444 ± 0 070	0 448 ± 0 050

Table IV Influence of Peptichemio on RCR, induced with ADP (1 × 10^{-4} M), adrenaline (1 × 10^{-4} M) and collagen (50 µg/ml) the values indicate the retraction (% + SD) in the 30th min of incubation at 37°C

PRP +	ADP	Adrenaline	Collagen
Michaelis buffer (pH 7 35), 5–30 min	77 6 ± 5 2	75 6 ± 4 3	83 1 ± 4 9
Peptichemio 15 µg/ml, 5 min	75 6 ± 5 2	74 5 ± 4 3	81 2 ± 5 3
Peptichemio 15 µg ml, 30 min	73 1 ± 3 3	72 8 ± 5 6	80 0 ± 3 8
Peptichemio 30 µg/ml, 5 min	68 5 ± 2 5	70 8 ± 5 1	78 7 ± 3 9
Peptichemio 30 µg/ml, 30 min	67 5 ± 2 8	68 9 ± 3 2	77 4 ± 4 5
Peptichemio 150 µg ml, 5 min	8 7 ± 7 8	1 2 ± 1 0	0 6 ± 0 6
Peptichemio 150 µg/ml, 30 min	4 1 ± 3 7	0 8 ± 0 5	0 6 ± 0 6

High concentration of Peptichemio (150 µg/ml) inhibited concurrently platelet aggregation, release of PF4, availability of PF3 and APh, as well as RCR, regardless of inducer used However, similar high concentrations cannot be reached in plasma of treated subjects Noteworthy was that APh and PF3 availability, although significantly decreased following the high dose of Peptichemio in the 20th min, were found to be increased ini-

tially If it is not an misleading phenomenon, the possibility of some acti vation of thromboplastic lipoprotein cannot be excluded, as APh were found to be an enzymatic 'marker' of the lipoprotein complex with PF3 activity [3] In this case a certain relationship to the frequent thromboses observed during Peptichemio therapy [5, 10, 13], can be traced, as the local concentration of the drug in the vein injected is undoubtedly higher than average therapeutic plasma concentration

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Hb Camden and Hb Hope Found During Routine Testing

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Key Words Hemoglobinopathies Hb Camden Hb Hope

Abstract The electrophoretically fast moving hemoglobin variant Hb Camden *(β 131 (H9) gln→glu) has been found in a middle aged female suffering from pul monary disease* and *Hb Hope (β 136 (H14) gly→asp) which migrates just ahead and very close to Hb A was present in a young obstetrical patient with a mild he* molytic anemia

Current interest in sickle cell anemia has led various institutions to routinely test their patients for possible hemoglobin (Hb) variants During such a program in Grady Memorial Hospital several variants other than Hb S have been found, two of these are described here

Methods

Blood was collected in vacutainer tubes containing EDTA Hematological eval uation was done by standard methods [1] Heat stability tests followed the proce dure of GRIMES *et al* [2] as modified by KLEIHAUER *et al* [3] Alkali resistant hemo globin was determined by the method of BETKE *et al* [4] Red cell hemolysates were prepared by standard procedures [5] Electrophoresis of the hemoglobin at pH 9 0 followed the starch gel electrophoretic method of EFREMOV *et al* [6] Quantitation of hemoglobin components was made by chromatography on col umns of DEAE Sephadex DEAE-cellulose and CM-cellulose [7 10] Larger amounts of each variant were isolated by the same procedures and structural anal ysis on this isolated material were made with methods described in detail before [11–13]

[1] On leave from the Department of Biochemistry, Faculty of Agriculture, University of Skopje, Skopje

Patients

A S, a 49 year old black female with 6 children was seen regularly in the Pulmonary Clinic for chronic pulmonary infiltrates There did not appear to be any adverse condition associated with her hemoglobin abnormality, a slightly ab normal hematology (table I) can probably be attributed to the pulmonary problem she had

B T, a 20 year old female, was first seen for abdominal pain during her last month of pregnancy She was released after being started on ferrous sulfate, and prenatal vitamins including folic acid One month later she delivered a normal 3 1 kg female, and further contact was lost after the initial postnatal visit

Results

Hematological data on both individuals are given in table I

Starch gel electrophoresis at pH 9 0 of the hemoglobin of A S showed an abnormal band moving ahead of Hb A in a position usually reserved for the J hemoglobins The hemoglobin variant in B T was not readily separable from Hb A by this electrophoretic technique, however, an additional zone was demonstrable by cellulose acetate electrophoresis with an anodal mobility slightly greater than that of Hb A

Separation of the variant of A S from Hb A was obtained by chromatography on DEAE-Sephadex, quantitative data are Hb A_2 2 4%, Hb A 51 1%, and Hb X 46 5% CM-52 cellulose chromatography also gave excellent separations Incomplete separation of the variant of B T from Hb A was obtained by DEAE-cellulose chromatography using a pH gradient varying from 8 3 to 7 5 [7, 8] Quantitative data are Hb A_2 3 1%, Hb A 51 5% and Hb X 45 4%

The stability of the hemoglobin of subject B T at 60 °C and at pH 6 9 was less than that of a normal control, and about 30–40% of the hemoglobin precipitated after an incubation of 5–10 min The heat stability of the hemoglobin of patient A S was normal

Structural studies of the abnormal β-chains of both variants resulted in the recovery of all tryptic peptides with the exception of the βT-12[1] peptide (residues 113–120 inclusive) In the case of A S the amino acid composition of all recovered peptides was as expected except for that of peptide T-13 Data given in table II suggest a replacement of a glutaminyl by a glutamyl because of a low recovery of ammonia Such a

Table I Hematology profile, determined with a Coulter Counter Model S

	Patient A S	Patient B T
WBC, $\times 10^3$	20 9[1]	7 3
RBC, $\times 10^6$	4 37	3 38
Hb, g%	11 3	9 4
PCV, %	35 0	28 1
MCV, μm^3	80 0	84 0
MCH, pg	27 0	27 5
MCHC, %	32 0	34 0
Platelets	increased	normal
Retics, %	0 7	1 3
RBC morphology	normal with few target cells	2+ anisocytosis, 2+ poikilocytosis
Hb F_{AD}, %	0 5	0 7

[1] Pulmonary disease (infiltrates)

substitution can occur either in position 127 or in position 131 Peptide T-13 was digested by chymotrypsin [14] and the resulting fragments rechromatographed on a column of Dowex 1-X2 A T-13 peptide isolated from a β^A-chain was digested under the same conditions and served as control A dipeptide, Gln Lys, was isolated from the digest of the normal T-13 and was eluted at pH 9 0 whereas a corresponding dipeptide from the digest of the T-13 of the abnormal β-chain was eluted at pH 6 8 Amino acid analysis of this peptide contained only a fragment of one ammonia residue indicating a Glu-Lys composition Thus, the amino acid replacement in this β-chain concerns a Gln→Glu substitution in position 131, similarly as has been observed in Hb Camden [15]

The amino acid composition of all tryptic peptides of the β-chain B T was as expected, except for that of the T-14 peptide Data from table II show that this βT-14 contained no glycyl residue (one in the normal βT-14) and two aspartyl residues (one in the normal βT-14) Comparison of the ammonia levels in hydrolysates of the *normal βT-14* and the βT-14 of B T suggests a replacement which concerns an aspartyl residue rather than an asparaginyl residue Such a substitution in position 136 (Hb Hope) has previously been identified [16]

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Marker Chromosome in Myeloproliferative Syndrome

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Key Words Chromosomal anomalies Karyotype Marker chromosome Myeloproliferative syndromes

Abstract Chromosomal aberrations in a case of atypic myeloproliferative syndrome are reported The analysis was carried out on short term cultures of sternal bone marrow with the ASG and fluorescence method 90% of the metaphases studied showed pseudodiploidy one of the chromosomes 12 was missing and one submedian marker chromosome of medium size and atypical morphology appeared

Chromosomal aberrations are frequent in myeloproliferative disorders Chronic granulocytic leukemia is the most important, which may be characterized also by other chromosomal anomalies in addition to the well known Philadelphia chromosome The first may be extremely various especially during the blastic transformation of the disease In other cases of myeloproliferative syndromes numerical aberrations are frequent but structural anomalies are also not rare With the exception of the Philadelphia chromosome, these chromosomal aberrations are not specific

In the present study we are reporting on the rare chromosomal aberration of a patient suffering from atypic myeloproliferative syndrome, which caused a considerable morphologic problem in addition to its unusual character

Case Report

J T 61 year-old patient One year prior to the present admission he was hospitalized at our department because of spondylarthrosis At that time the differential

and quantitative blood count was normal During the screening prior to the present admission the number of white blood cells was 64 000/μl With the exception of moderate articular pain he was free from complaints

Slightly pale mucous membranes Lymph nodes are not observed Liver exceeds by 5 cm, spleen by 10 cm the costal arch Packed cell volume 37%, hemoglobin 10 9 g%, leukocytes 58 000/μl Differential blood count 3% myeloblasts 1% promyelocytes 2% myelocytes 30% juveniles 30% stabs, 28% segmented 3% eosinophils 3% lymphocytes Platelet count 160 000/μl Leukocyte alkaline phosphatase score 335 97% positive cells Bone marrow adequate cell content, myelopoesis in the foreground All members of the myeloid line are represented with dominance of mature forms The number of normoblasts is somewhat lower than usual The number and form of megakaryocytes are normal Serum uric acid 7 6 mg% The other laboratory examinations yielded normal data

The patient received initially Myleran then Myelobromol without success the number of white blood cells increased progressively to 100 000/μl parallel with the enlargement of the liver and spleen Following the ineffective cytostatic treatment X ray therapy was introduced for the liver and spleen in a dose of 900 rad each As a result the spleen became smaller and the number of white blood cells diminished to 30 000/μl Remission was very short and soon we observed the same leukocyte count as prior to irradiation Upon Zytostop (1 2 5, 6 tetra methan sulfonyl D mannitolum) medication the count improved again and with a dose of 300 mg weekly the leukocyte count could be maintained at about 30 000/μl At that time the thrombocyte count was 80 000/μl and the patient requires about 1 000 ml transfusion every month During the observation period of 1 year the general condition did not change significantly

Methods

Chromosome analysis was carried out three times using a 24 hour bone marrow culture Chromosome preparations were made according to the technique of KIOSSOGLOU *et al* [4] with slight modifications The preparations were examined with Giemsa stain respectively ASG and fluorescence method [3 11]

Results

Although the examinations were repeated three times, only preparations of relatively poor quality were obtained, which may be assigned to the unusually strong contraction It was not possible to prevent this by reducing the dose of Vinblastin to one-tenth of the usual quantity We karyotyped 20 metaphases and found 18 to be pseudodiploid Upon applying conventional Giemsa stain we observed a loss in the C group and an additional marker chromosome which was similar in size to the D group The

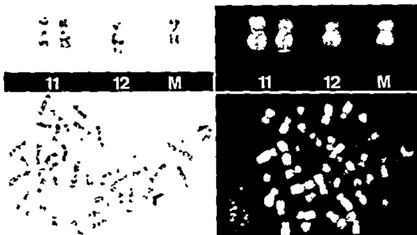


Fig 1 Metaphases stained with the ASG and fluorescence method The arrows *point to the characteristic marker chromosome Above chromosomes* 11 and 12 originating from the same metaphases as well as the marker (M) may be seen

marker had nearly median centromere and not using the banding technique, seemed to be an isochromosome Following the application of the ASG and fluorescence method, one of chromosome 12 could be identified as the missing chromosome of the C group and it became evident that the marker was no isochromosome (fig 1) It had clear submedian centromere and its homogenity was obviously shown by the fluorescence technique A similar, but not so distinct homogenity was observed with the ASG method

Discussion

Our case is unusual from a clinical and cytogenetical viewpoint Despite the detailed clinical examinations and an observation period of 1 year we did not succeed in diagnosing a more limited disease entity than the myeloproliferative syndrome The abundant quantity of bone marrow obtained at the time of repeated sternal punctures, the unfavorable response to cytostatic therapy, as well as the chromosomal aberration reveal that our case is closest to chronic granulocytic leukemia On the oth-

er hand, the consistently high leukocyte alkaline phosphatase is indicative for an other form of myeloproliferative conditions

From a cytogenetic point of view, chronic granulocytic leukemia is the most important myeloproliferative disorder. In the majority of cases this condition is characterized by the well-known Philadelphia chromosome, on the other hand, in its absence, other chromosomal aberrations are not infrequent. In the course of blastic transformation, the cytogenetic pattern becomes similar to that of acute leukemia. In this case the double or triple Philadelphia chromosome is the most typical in addition to the numerical aberrations. The development of isochromosomes is also comparatively frequent, and concerns generally one of the long arms of chromosome 17 or 18 [1, 5, 8, 10]. Using the banding technique LOBB *et al* [6] demonstrated recently in three cases the presence of the isomarker mentioned above, and assumed that this phenomenon is generally more frequent in tumorous diseases Similar conclusions might possibly be drawn from the isomarkers noted in other malignant diseases, as in malignant lymphoma [2, 9] and acute myeloid leukemia [7]

Morphologically, our case comes near to, but is undoubtedly no isomarker. The application of modern methods played a fundamental role in differentiation and the principal aim of presenting our case was to draw attention to this fact. We believe that the banding techniques are especially significant in the diagnosis of marker chromosomes. In addition, our data also indicate that a certain care is necessary in the estimation of the isomarkers mentioned earlier in the literature, and that with respect to tumorous diseases, the understanding of the significance and substantial role of isomarkers requires further detailed investigations

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EDMUND L. DUBOIS (ed.) **Lupus erythematosus** A Review of the current Status of Discoid and Systemic lupus erythematosus and their Variants University of Southern California Press Los Angeles 1974 763 pp., US $ 36.00

This book, with many other collaborators in addition to DUBOIS gives general and detailed information about all types of lupus erythematosus with many black and white figures and some excellent tables and color illustrations For the hematologist the chapter on immunologic phenomena in patients with systemic lupus erythematosus is of particular interest The bibliography lists 2 975 (!) publications

G ROSENOW, *New York N Y*

J D BAUER, PH G ACKERMANN, GELSON TORO **Clinical Laboratory Methods** 8th ed Mosby, St Louis 1974 947 pp., 497 fig., 16 color plates, US $ 20.00

This new edition has been greatly expanded It contains many new laboratory techniques with chapters on e g, screening methods of hemoglobinopathies ferrokinetics and isotope techniques for intrinsic factors The section on WBC pathology includes among many other techniques the muramidase assay, and numerous other new techniques for examination of hematologic disorders The methods for automatic blood cell counting are mentioned but not described in detail The book is particularly valuable for technicians in clinical laboratories

G ROSENOW *New York N Y*

Acta haemat 54 65–74 (1975)

Characterization of Ineffective Erythropoiesis in Erythroleukaemia[1]

W. QUEISSER, U PEPPERL, U KEMPGENS and U. MÜLLER

I Department of Internal Medicine, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Heidelberg

Key Words Autoradiography Cytophotometry DNA synthesis Erythroblasts Erythroleukaemia G_0 cells Ineffective erythropoiesis

Abstract In 6 cases initially showing a symptomatology of erythroleukaemia, the proliferation of erythroblasts was studied by combined Feulgen cytophotometry and autoradiography after labelling with ^{3}H TdR *in vivo* A proliferation disturbance was observed consisting of an accumulation of diploid and unlabelled cells and a decreased proportion of cells in S This defect occurred within the basophilic cell compartment in one case and within the early polychromatic cell compartment in all 6 cases The results indicate the existence of out-of cycle cells, which may be responsible for the inefficacy for erythropoiesis in this disease The defect was present in all 4 cases with acute erythroleukaemia. In one of the two cases showing no deterioration of the physical and haematological condition over a period of years, an additional defect was observed, consisting of a striking accumulation of tetraploid unlabelled cells Therefore, the technique used may be suitable as a diagnostic tool for evaluation of new types of ineffective erythropoiesis

The erythroblastic hyperplasia, low absolute reticulocyte count and anaemia in typical cases of erythroleukaemia are due to reduced or ineffective red cell production To describe this defect within the erythrocyte precursor compartments, *in vivo* labelling with tritiated thymidine (^{3}H-TdR) in patients with Di Guglielmo's disease was done [4, 5, 10] A continuous decrease of the labelling index from normal or subnormal values within the basophilic erythroblasts to always lowered values in the po-

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lychromatic cells was observed, suggesting a proliferation disturbance mainly limited to the polychromatic precursor cell compartment The DNA synthesis time of the basophilic as well as of the polychromatic cells showed no marked prolongation as it has been described in one case [12] To explain this discrepancy, the presence of two different cell fractions was discussed, the large one showing a lack of the dividing activity (out-of cycle cells) and the small one showing a 'normal' cell cycling

In order to gain further information about this proliferation disturbance, in the present study the erythropoietic cell proliferation was investigated by combined application of cytophotometric determination of the DNA content and autoradiographic labelling with ^{3}H-TdR. The study was performed in a group of 6 patients showing a characteristic symptomatology for erythroleukaemia at the beginning of the disease Other conditions as vitamin B_{12} or folic acid deficiency or sideroachrestic anaemia were excluded In 4 patients the diagnosis of acute erythroleukaemia was confirmed, and the other two patients showed a chronic disease of unknown origin

Table I Haematological data and bone marrow differentiation

	Case 1 *1897, m (Freiburg)	Case 2 *1928, m (Tübingen)	Case 3 *1925, f (Freiburg)	Case 4 *1892, m (Ulm)	Case 5 *1904 m (Mainz)	Case 6 *1900, m (Ulm)
Hb, g/100 ml	7 6	10 2	7 9	7 8	8 3	9 1
RBC, $\times 10^6/\mu l$	2 25	2 95	2 50	2 50	2.90	2 80
HKT, %	23	31	28	31	30	34
MCH, pg	34	34	32	31	29	32
MCV, μm^3	102	105	112	124	103	121
Reticulocytes/mm^3	4,500	5,900	82,500	95 000	72,500	84 000
Platelets/mm^3	73,000	255,000	130,000	26 000	15,000	110 000
WBC/mm^3	1,760	8 400	5 000	19,700	2 230	3,300
LBC, %	25	55	0	47	0	0
Erythroblasts/100 WBC	–	3	100	13	0	1
Bone marrow						
Erythroblasts, %	56	65	55	77	75	80
Sideroblasts	++	+++	+++	++	+++	+
PAS-positive erythroblasts	+++	+	∅	+++	+++	+
LBC, %	16	18	28	8	9	0

Material and Methods

Patients Six patients were studied. The main haematological data are given in table I Though the initial symptomatology was consistent with the diagnosis of Di Guglielmo's disease in all patients, only in cases 1–4 deterioration of the clinical and haematological condition appeared rapidly, and the patients died within 11 months, indicating the diagnosis of acute erythroleukaemia. Case 5 was firstly admitted at hospital in January, 1966 At that time the diagnosis of erythroleukaemia was suspected from a mild jaundice, pancytopenia, a marked erythroblastic hyperplasia and moderate blast cell infiltration of the bone marrow The patient was treated symptomatically and was followed over a period of 6 years without being treated by chemotherapeutic agents Case 6 was admitted in July, 1970, showing a severe anaemia and moderate thrombo- and leucopenia. The number of erythroblasts of the bone marrow was increased, and the cells showed striking megaloblastic nuclei. However, the Schilling test was normal He was treated by folic acid, vitamin B_{12} and also by thymidine infusion without any success. The patient, who had to be transfused repeatedly, was followed over a period of 2.5 years and the physical and haematological state remained unchanged. The patients were completely informed about the investigation procedure and agreed to it. From the diagnosis mentioned, a shortened life expectancy had to be suspected.

General procedure In all 6 cases 0.1 μCi ^{3}H TdR/kg body weight (specific activity 2 Ci/mM) was given intravenously 1 h after the injection, marrow was aspirated into a syringe containing 0.5–1.0 ml EDTA (1.1% Na_2EDTA in 0.7% NaCl) Smears were made from the marrow spicules and stained with Pappenheim stain. Optimal cell areas were marked, and cells were photographed (Agfachrome L 5) for subsequent localization for consecutive cytophotometry and autoradiography Thereafter, the stain was leached out by treatment with 50% ethanol, and the smears were restained by the Feulgen method applying pararosanilin for Schiff's reagent (hydrolysis for 12 min in 1 n HCL at 60 °C, staining time 45 min)

Cytophotometry For the determination of the DNA content, a Cytoscan 05 (Zeiss, Germany) was used. Monochromatic light at 570 nm was employed, the diameter of circular area of the photometric field being 1 μm. The extinction (E) was measured at 10 points within the nucleus. The nuclear area (A) was determined from the nuclear diameters, which were measured by an ocular micrometer The relative DNA content (AU = arbitrary units) of individual nuclei was calculated from AU = E × A.

Autoradiography Autoradiographs of the Feulgen-stained smears were made by the dipping film technique using Kodak L 5 liquid emulsion [1] The smears were exposed for 40–60 days. The background was determined from the fraction of diploid erythroblasts. *Cells showign more than 3–5 grains were considered as labelled.*

Cytology The subclassification of the erythropoietic cells was performed in panoptic stain According to a morphologic classification described in a previous study

[1] The autoradiogrpahy was performed by PD Dr D HOELZER, Center of Clinical Research Department of Clinical Physiology University of Ulm, Ulm (FRG).

11, the following cell types were distinguished basophilic erythroblasts (E_{1-3}), early polychromatic erythroblasts (E_4) and late polychromatic erythroblasts (E_5) Apart from the cytoplastic staining properties cells showing a nuclear area smaller than 20 μm^2 were designated as E_5 Polynuclear cells or cells of other atypical nuclear formation were not included into the study

Results

The relative DNA values were summarized in cytograms showing the distributional pattern of DNA in each cell type For evaluation of the diploid standard (2c), the DNA values of the early polychromatic erythroblasts being not labelled with ^{3}H-TdR have been averaged The mean tetraploid value (4c) was calculated from 2c By the technique described, within a proliferating cell population diploid cells showing no labelling

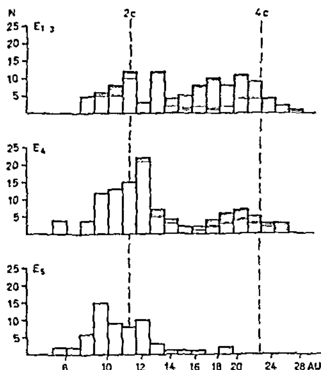


Fig 1 Relative DNA content in arbitrary units (AU) and ^{3}H TdR labelling (shaded areas) of erythroblasts in acute erythroleukaemia (case 3) Semilogarithmic scale N = Number of cells, 2c = diploid standard, 4c = tetraploid standard, E_{1-3} = basophilic, E_4 = early polychromatic and E_5 = late polychromatic erythroblasts

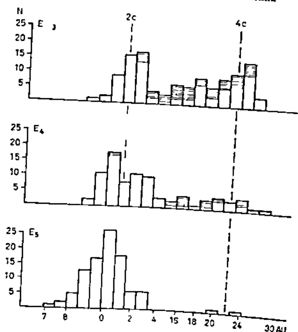


Fig 2 Relative DNA content in arbitrary units (AU) and ³H TdR labelling (shaded areas) of erythroblasts in case 5 Legend see figure 1

are assumed to be in the postmitotic rest period (G_1) or to be out of cycle (G_0) Unlabelled tetraploid cells are in the premitotic rest period (G_2) and the ³H TdR labelled cells in the DNA synthesis period (S) The unlabelled cells with a DNA content between the diploid and tetraploid level, which cannot be attributed to one of the resting periods are designated as U cells (U = unrecognizable)

In the *basophilic erythroblasts* (E_{1-3}) of cases 1–6 the DNA values are distributed from the diploid to the tetraploid level including a considerable proportion of labelled cells indicating the presence of a complete cell cycle Characteristic cytograms are given in figure 1–3 The fractions of basophilic erythroblasts in the different cell cycle stages are compared with data obtained from four normal humans (table II) and show a normal proportion of cells in G_1, S and G_2 in 4 cases In cases 1 and 5 the proportion of cells in G_1 is elevated (57 and 39%) and the proportion of cells in S is markedly decreased (23 and 26%) The number of cells in G_2 and in U is somewhat higher than in normal humans.

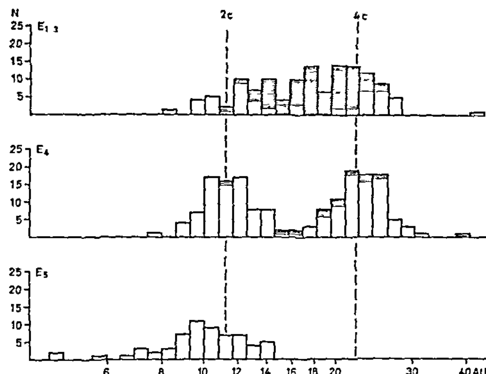


Fig 3 Relative DNA content in arbitrary units (AU) and ³H TdR labelling (shaded areas) of erythroblasts in case 6 Legend see figure 1

The *early polychromatic erythroblasts* (E_4) show a striking elevation of unlabelled diploid cells as seen in figures 1 and 2 The fraction of these cells ranged from 46 to 72% Correspondingly, a reduction of cells in S from 7 to 26% was observed (table II) The proportion of cells in G_2 was normal except for case 6, which showed a second accumulation of unlabelled cells within the tetraploid range (fig 3)

Discussion

In 6 patients who initially showed a clinical symptomatology characteristically for acute erythroleukaemia, the proliferation of erythroblasts in the bone marrow was studied using the combined application of cytophotometric determination of the DNA content and ³H-TdR autoradiography *in vivo* The results of further autographic investigations are reported elsewhere [5] The main pathological finding of the present study

Table II Percentage distribution of erythroblasts in the different DNA synthesis stages (G_1, S, G_2)[1]

Case No	Basophilic erythroblasts (E_{1-3})					Early polychromatic erythroblasts (E_4)					Late polychromatic erythroblasts (E_5)				
	N	G_1	S	G_2	U	N	G_1	S	G_2	U	N	G_1	S	G_2	U
1	124	57	3	16	4	104	67	7	12	5	101	98	0	0	2
2	177	27	53	17	3	107	63	20	13	4	60	98	0	0	2
3	108	36	49	12	3	116	66	16	15	3	61	97	0	0	3
4	116	21	76	3	0	151	67	26	4	3	22	0	0	0	0
5	119	39	26	33	2	86	72	13	13	2	111	98	0	0	2
6	129	20	59	18	3	169	46	7	44	3	61	100	0	0	0
Normal values		28 (25–37)	63 (51–70)	8 (5–11)	1 (0–1)		19 (8 30)	65 (55–75)	15 (5–26)	1 (0–1)		96 (95–100)	4 (0–6)	0	0

[1] Normal values from QUEISSER *et al* [11] N = Number of cells, U = unrecognizable cells

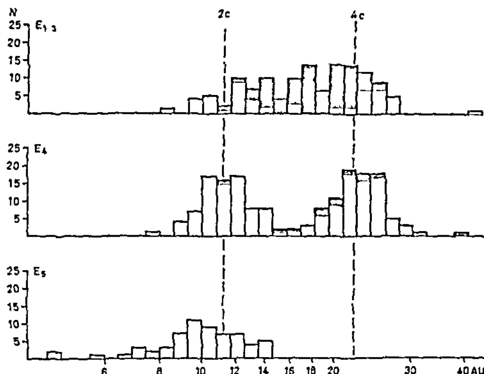


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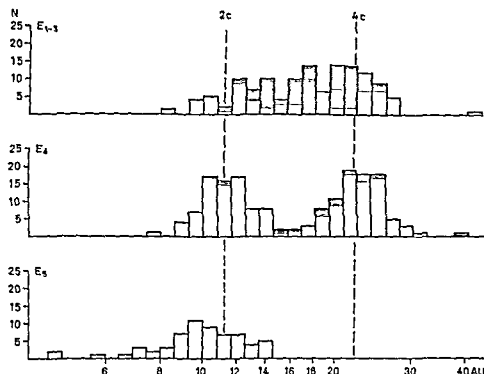


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[1] Normal values from QUEISSER *et al* [11] N = Number of cells, U = unrecognizable cells

was a considerable proportion of unlabelled diploid cells, which occurred within the basophilic cell compartment in one case and within the early polychromatic cell compartment in all 6 cases Consequently, a decreased proportion of ^{3}H TdR-labelled cells representing the DNA synthesis stage was observed

From the data obtained it cannot be decided whether the diploid unlabelled cells are in cycle (G_1) or out of cycle (G_0) The DNA synthesis time studied in 4 of our cases by labelled mitosis curve or by the double labelling technique showed no striking prolongation [5] Therefore, it is suggested that the inefficacy of red cell production results from a fraction of diploid cells being out of cycle (G_0) A direct method for calculation of the proportion of G_0 cells from *in vivo* ^{3}H-TdR autoradiography and combined cytophotometry and autoradiography was established by ourselves [9] However, in the present study the data for this calculation were not available In cases of acute leukaemia it has been demonstrated that the cells being out of cycle are capable to re-enter the cycle [2, 7] By continuous ^{3}H-TdR infusion over a period of 8–10 days, an increase of labelled leukaemic blast cells up to 90% was observed [1] From these observations, a re entrance of the G_0 blast cells into the cell cycle after a lag period of days was suggested Therefore, in cases of erythroleukaemia, the early polychromatic erythroblasts being diploid and not labelled may be capable to re enter the cycle Additionally, a premature cell death of nucleated cells within the bone marrow may contribute to the inefficacy of leukaemic erythropoiesis

The proliferation of erythroblasts in erythroleukaemia using ^{3}H-TdR autoradiography *in vivo* or *in vitro* was at first studied in two cases by GAVOSTO *et al* [3], later on in one case by WICKRAMASINGHE *et al* [13] and in another case by TODO [12] Except the case of WICKRAMASINGHE *et al* showing a normal labelling index in the basophilic as well as in the early polychromatic cells the labelling was markedly reduced A proliferation disturbance similar to that observed in the present study was suggested in preleukaemia [10] and acute leukaemia [6] This defect was found to be reversible during complete remission of acute leukaemia [8] No remarkable differences of the proliferation pattern of erythroblasts in preleukaemic condition, acute leukaemia and in acute erythroleukaemia could be established

As mentioned above, from the 6 cases initially showing the symptomatology characteristically for erythroleukaemia, only 4 cases progressed

Acta haemat. 54 75–81 (1975)

Pentose Shunt, Phosphoribosylpyrophosphate Generation and Purine-Phosphoribosyltransferases in Erythrocytes of Patients with Polycythemia vera[1]

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Key Words Adenine incorporation Adenine phosphoribosyltransferase Erythrocyte metabolism Pentose shunt Phosphoribosylpyrophosphate Polycythemia vera

Abstract Erythrocytes of patients with polycythemia vera exhibited increased activity of oxidative pentose shunt, accelerated generation of phosphoribosylpyrophosphate increased incorporation of ^{14}C-adenine into nucleotides and increased activity and thermostability of adenine phosphoribosyltransferase These abnormalities are attributed to age dependency of the pathways concerned and presence in polycythemia vera of an erythrocyte population younger than normal

Increased activity of several age-dependent enzymes in erythrocytes of patients with polycythemia vera (PCV) has been reported and attributed to the presence of an erythrocyte population younger than normal [1] In the course of studies on purine metabolism of the erythrocyte we observed increased adenine incorporation into its nucleotides in a gouty patient with PCV Since this pathway was found to be normal in patients with primary metabolic gout [11], a study of this and connected pathways and their possible age dependency was undertaken in subjects with PCV

[1] Supported in part by grant No 185 from the Chief Scientist of the Ministry of Health Israel

[2] Established investigator of the Ministry of Health of Israel

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Table I. Activity of metabolic pathways in erythrocytes[1]

Subjects	^{14}C-adenine incorporation into erythrocyte nucleotides nmoles/ml packed cells/min	PRPP generation nmoles/ml packed cells/min	Oxidative pentose shunt activity ($^{14}CO_2$ release) nmoles/g Hb/h
Control n = 18	1.48 ± 0.22 (1.08 – 1.87)	1.53 ± 0.24 (1.05 – 1.87)	415 ± 90.5 (242 – 555)
PCV n = 16	2.00 ± 0.40 (1.41 – 2.79)	1.85 ± 0.37 (1.02 – 2.52)	499 ± 152.5 (264 – 734)
	p < 0.001	p < 0.01	p < 0.001

[1] The values, given as mean ± 1 SD, are corrected to a mean corpuscular hemoglobin concentration of 32%; ranges are given in parentheses.

Table II. Enzyme activity in erythrocytes[1]

Subjects	Enzyme activity, nmoles/mg Hb/h		
	APRT	HGPRT	PRPP synthetase
Control n = 20	30.3 ± 4.54 (23.6 – 42.0)	102.4 ± 10.26 (88.6 – 131.6)	16.0 ± 2.65 (11.6 – 23.9)
PCV n = 16	33.1 ± 7.97 (21.9 – 49.3)	93.9 ± 14.2 (73.7 – 126.0)	15.4 ± 3.7 (9.0 – 22.8)
	p > 0.05 > 0.1	p > 0.05 < 0.1	p > 0.5

[1] As in legend to table I.

patients studied. On the other hand, high activity of PRPP synthetase was found in two PCV patients only and of HGPRT in only one PCV patient. The activities of APRT and HGPRT in the PCV patients exhibited a positive correlation ($r = +0.58$, $p < 0.025$).

Activity of enzymes and adenine incorporation in density-fractionated erythrocytes. The activity of APRT, PRPP synthetase and to a lesser extent also that of HGPRT exhibited age dependency, being higher in younger erythrocytes both in those from the six normal subjects and those from the four PCV patients studied (table III). In the erythrocytes of the

Of interest is the finding of increased heat stability of PCV erythrocyte APRT Increased APRT thermostability has been reported also for erythrocytes of HGPRT-deficient subjects and was ascribed to stabilization of the enzyme by augmented PRPP concentration [2] In view of the increased PRPP generation in erythrocytes of PCV patients it is probable that also in them the erythrocyte APRT stabilization is due to increased PRPP availability

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Parallel Tubular Structures in Lymphocytes

II Correlation with Cellular Immunity and Cytomegalovirus and Epstein-Barr Virus Antibodies in Hodgkin s Disease

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Key Words Cellular immunity CMV antibodies EBV antibodies Hodgkin s disease Lymphocyte inclusions Tubular structures in lymphocytes Ultra structure of lymphocytes

Abstract The increased incidence of parallel tubular structures in lymphocytes of patients with Hodgkin s disease was investigated for a correlation with either impairment of cellular immunity (measured by DNCB skin test and PHA induced lymphocyte stimulation *in vitro*) or an increase of antibodies against cytomegalovirus or Epstein Barr virus No correlations were found Statistical analysis revealed antibody titers especially in the group of patients with high percentages of lymphocytes containing the tubular inclusions This probably reflects only the connection between these findings and the progression of the disease The nature and function of the parallel tubular structures have to be investigated further In Hodgkin s disease they may have significance for the understanding of an alteration in lymphocyte function and morphology

The role of the lymphocyte in Hodgkin's disease is the subject of many investigations Cellular immunity may be impaired in early stages of the disease [4] while the humoral immune response can be decreased only in the later stages Defective T cell function has been assumed [5, 21] An increase of immunoblasts in the peripheral blood correlating with the activity of the disease has been described and interpreted as an immunological reaction to the disease process [10] Apart from these immunoblasts other varieties or morphological abnormalities of lymphoid cells in the peripheral blood have not been observed In ultrastructural studies on cells of the spleen and lymph nodes in Hodgkin s disease DORFMAN *et al*

[3] found parallel tubular structures in lymphocytes These structures have also been found in peripheral blood lymphocytes of healthy persons [14] and in some diseases [6, 13, 15, 17] In Hodgkin's disease an increase of the inclusions per cell and in the percentage of lymphocytes from the peripheral blood containing them has been noticed [9] Correlations with the histological classification or the clinical stage of the disease could not be established Mean values of lymphocytes with the parallel tubular structures were increased for all groups of Hodgkin's patients, but especially for the histologically and clinically unfavourable groups To assess the significance of these parallel tubular structures in the lymphocytes a study was done on a possible correlation between the percentage of abnormal lymphocytes and some parameters for cellular immunity and of cytomegalovirus (CMV) and Epstein Barr virus (EBV) antibody titers in patients with Hodgkin's disease Antibodies to both viruses were found to be significantly elevated in Hodgkin's disease [19]

Methods and Materials

A number of patients with Hodgkin's disease from the group previously described [9] were investigated. Histological classification was done according to the Rye criteria by Prof A ARENDS, and Dr R. EIBERGEN (Department of Pathology) Clinical investigation included a laparotomy with splenectomy and the use of our own classification [8] in addition to the Ann Arbor modification [2] for the clinical staging of the disease

DNCB and PHA DNCB sensitization and evaluation were performed according to the method described by BLEUMINK *et al* [1] PHA stimulation of lymphocytes *in vitro* was measured by the methods of DE GAST *et al* [5]

EBV and CMV antibodies Indirect immunofluorescence tests for both antibodies [12, 18] were modified as described previously [19]

Statistical analysis The Wilcoxon paired sample rank test was used for testing the differences in DNCB skin test scores, PHA stimulation of lymphocytes *in vitro* and EBV and CMV antibodies titers in sera of patients with age and sex matched controls (healthy blood donors) Antibody titers less than 1 8 for CMV and 1 10 for EBV were recorded as negative

Results

Clinical stages, histological classification, PHA-stimulation, DNCB-score and CMV- and EBV-antibody titers are summarized in table I As can be seen no correlation could be found for each individual patient between the immunological status as measured by PHA-stimula-

Table I Histological classification, clinical staging, percentage of lymphocytes with parallel tubular structures, CMV- and EBV-antibody titers and parameters for cellular immunity

Patient No	Histology	Staging: Groningen 1972	Staging: Ann Arbor	Percentage tubular structures	Antibody titers[1]: CMV	Antibody titers[1]: EBV	PHA[1] cpm	DNCB[1]
1	LP	loc	CSIA $PSI_{m\ b-e}$	10	8(neg)	640(1,280)	33,526(21 890)	11+ (6+)
2	LP	loc	CSIA $PSI_{m\ b-e}$	14	neg(32)	320(1,280)	30,495(25 874)	3+(12+)
3	NS	loc	CSIA $PSI_{m\ b-e-}$	5	512(64)	640(320)		
4	LP	loc	CSIA $PSI_{m\ b-e}$	23	512(neg)	5,120(320)		
5	NS	loc	CSIA $PSI_{m\ b-e}$	20	neg(neg)	640(640)	18,533(32,418)	5+(11+)
6	NS	loc	CSIIA $PSII_{m\ b\ e}$	7	512(256)	1,280(320)	23,334(18,905)	4+(12+)
8	NS	*loc*	*CSIIA* $PSII_{m\ b-e}$	*12*	*256(128)*	*1,280(1,280)*		
9	LD	haem diss	CSIIA $PSIII_{m\ b\ e+}$	32	256(128)	2,560(640)	31,690(17,100)	5+(10+)
12	LD	haem diss	CSIIA $PSIII_{m\ b\ e+}$	10	128(128)	640(neg)	43,868(11 084)	1+(12+)
14	MT	haem. diss	CSIIIB $PSIII_{m\ b-e+}$	9	neg(neg)	160(neg)	35,943(21,937)	9+(10+)
15	LD	haem diss	CSIIIB $PSIII_{m\ b-e+}$	12	512(512)	160(320)	10 062(23 908)	12+(12+)
16	MT	haem diss	CSIIIB $PSIII_{m\ b\ e+}$	8	16(32)	1,280(1,280)		
18	LD	haem diss	CSIIIB $PSIII_{m\ b\ e+}$	44	512(16)	1,280(neg)		
19	LD	haem. diss	CSIIIB $PSIII_{m\ b\ e+}$	42	2,048(16)	1,280(640)		
20	LD	haem diss	CSIIIB $PSIII_{m\ b-e+}$	31	512(8)	2,560(1,280)	10,992(28,754)	1 5+(12+)
22	LD	haem diss	CSIIB $PSIV_{m+b}$	8	512(128)	160(1,280)		
23	MT	haem diss	CSIIIB $PSIV_{m+b-e+}$	14	128(512)	2 560(160)	1,759(23,685)	0+(11+)
24	NS	haem diss	CSIIBL $PSIV_{m+b}$	23	128(16)	2,560(320)		

LP = Lymphocytic predominance, NS = nodular sclerosis, MT = mixed type, LD = lymphocytic depletion, loc. = localized Hodgkin's disease, haem diss = haematogenous disseminated Hodgkin's disease, neg = negative (<1 8 for CMV titer, <1 10 for EBV titer), cpm = counts per minute

[1] Age and sex matched control

tion and DNCB score and the percentage of lymphocytes with tubular structures Patients with a low percentage of tubules containing lymphocytes (<15%) had only a tendency for a decreased DNCB score as compared to normals (p = 0 10) while no significant differences were found with the matched controls in the PHA-stimulation test The number of patients in the group with a high percentage of abnormal lymphocytes (>15%) was too small for statistical evaluation Also, for the individual patient no correlation could be established between the presence of tubular structures in the lymphocytes and the titers of CMV- or EBV-antibodies

The statistical analysis of differences in virus titers between the patient

Table II. Statistical analysis (Wilcoxon paired sample rank test) of differences in virus titers, PHA in vitro stimulation of lymphocytes and scoring of DNCB skin tests between groups of patients and controls

	CMV		EBV		PHA		DNCB	
	No	sign.	No	sign.	No	sign.	No	sign.
Low	6	NS	5	NS	4	–	4	–
[illegible]	8	p = 0.10	10	p < 0.05	6	NS	5	p = 0.10
par. lymph. < 15%	8	NS	9	NS	7	NS	6	p = 0.10
par. lymph. ≥ 15%	6	p = 0.05	6	p = 0.05	3	–	3	–
Total	14	p < 0.05	15	p < 0.05	10	NS	9	p < 0.02

par. lymph. = Parallel tubular structures containing lymphocytes. No = number of pairs used for the Wilcoxon test. NS = not significant.

groups with low or high percentages of abnormal lymphocytes and their matched controls is given in table II. CMV-antibodies were significantly increased in the total group and the subgroup with high percentages of lymphocytes with parallel tubular structures, but not in the subgroup with a low percentage of abnormal lymphocytes. EBV-titers were raised in the whole group of patients investigated (p ~ 0.05) primarily due to a raise in titers in the group with a high percentage of abnormal lymphocytes.

Discussion

The parallel tubular structures in lymphocytes can occur in normal as well as in several diseases [6, 13–15, 17]. The increase in Hodgkin's disease could have a relation with the known functional abnormality of the lymphocyte in this disease [21]. A correlation between the increased number of inclusions containing lymphocytes from Hodgkin patients and the histological or clinical classification could not be shown [9].

A correlation with the immune status as measured by DNCB-score and PHA-stimulation was also lacking. For the impaired cellular immunity the number of patients with high percentages of abnormal lymphocytes was too small for statistical evaluation of this group. However, this impairment increases with the histological and clinical progression of the disease [4, 5], which was also found for the increase in lymphocytic tubular structures [9]. Therefore it would hardly be surprising if the morphologi

cal abnormality of the lymphocyte also parallels the decrease of cellular immunity Our investigations did not indicate that impaired lymphocyte function as the cause for decreased cellular immunity had a connection with the morphological alterations (patient No 2, 6, 12, 15, 23) Another possibility that was investigated considered a correlation of the increase in parallel tubular structures with the elevation of virus antibodies described previously [19] Several authors have mentioned other inclusions, the tubular-reticular structures in cells and their connection with viral infections, especially in SLE [6, 7, 11] GOODMAN *et al* [6] also observed the incidence of the parallel tubular structures in their material, but did not consider them to be of any significance for their investigations The possible role of viral infections, especially EBV in Hodgkin's disease has been put forward [16, 19, review 21], although the findings of LANGENHUYSEN *et al* [19] may be interpreted as indicating that the prevalence of antibodies to latent viruses reflects a humoral hyperreactivity compensating for a decrease in cellular immune competence In our investigation it could not been shown that in the individual patient lymphocytic abnormalities were connected with an elevation of antibodies to CMV or EBV The statistical correlations found possibly merely reflect the connections between the disease progression, decrease of cellular immunity and raise in antibody titers as found by LANGENHUYSEN *et al* [19] But, although hardly probably, a more direct relation between the morphological alterations in the lymphocytes and virus infections cannot entirely be excluded yet

The possibility that the increased inclusions in the lymphocytes of patients with Hodgkin's disease represent a structural damage whether due to a disturbance of metabolism or to an outside agent, deserves further investigation The decrease of cellular immunity as caused by such a mechanism has also to be explored further

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Role of Transcobalamins I, II and III in the Transfer of Vitamin B12 to Human Bone Marrow Cells *in vitro*

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Key Words Bone marrow cells Metabolic inhibitors Transcobalamins Vitamin B_{12} metabolism

Abstract A study of the uptake of transcobalamin bound ^{57}Co-cyanocobalamin by suspensions of human bone marrow cells has indicated that these cells can take up vitamin B_{12} from all 3 transcobalamins (I, II and III) Similar transport processes were involved in the uptake from the 3 transcobalamins, uptake was dependent on the presence of calcium ions cellular respiration and free sulphydryl groups These results suggest that contrary to current belief, all 3 transcobalamins play a role in the transfer of vitamin B_{12} to tissue cells

The vitamin B_{12}-binding proteins present in normal human serum can be separated chromatographically into two main fractions which are termed transcobalamins I and II [15] Transcobalamin I (TCI), a glycoprotein [1] which probably originates from neutrophil granulocytes [21] and their precursors [24], is antigenically similar to the 'R' binders present in tissue fluids [10, 11, 20] By contrast, transcobalamin II (TCII) is not a glycoprotein [2], is synthesised in the liver [6, 7, 23], and is antigenically different from the 'R' binders [11, 22] A third binder, termed transcobalamin III (TCIII), has recently been described in normal human serum [4, 12, 13] This binder has the same molecular radius and antigenic characteristics as TCI [8] and may represent a fraction resulting from a partial separation of the 3 components of TCI with different iso electric points [17] In a recent study, WICKRAMASINGHE and MOFFATT [25] showed that the uptake of serum bound ^{57}Co-cyanocobalamin by freshly-aspirated human bone marrow cells was mediated via an active, calcium-dependent process requiring the presence of cellular respiration and free sulphydryl groups In this paper, we report the re-

sults of a study of the relative importance of TCI TCII and TCIII in the transfer of vitamin B_{12} to human bone marrow cells *in vitro* In addition we present data on some of the factors which influence these transport processes

Materials and Methods

Fractions containing TCI TCII and TCIII were prepared from each of 5 samples of sera obtained from healthy group AB subjects The sera were saturated with ^{57}Co vitamin B_{12} by the addition of 2 ng of ^{57}Co-cyanocobalamin (specific activity 100–300 μCi/μg)/ml of serum the unbound vitamin B_{12} was removed by dialysis and the samples were then fractionated by a combination of DEAE cellulose chromatography and Sephadex G 200 filtration [4 8]

Short term marrow cultures were prepared using marrow aspirates from 7 patients with normoblastic erythropoiesis The freshly aspirated bone marrow was mixed with 5 ml Hanks solution containing preservative free heparin This mixture was forced through a 21 gauge needle once and a 25 gauge needle twice and the resulting cell suspension was centrifuged at 1 100 *g* for 5 min The buffy coat was separated washed 3 times in Hanks solution and finally resuspended in 4–8 ml Hanks solution Equal volumes of this cell suspension were mixed with an appropriate volume of radioactive TCI TCII or TCIII such that the same amount of radioactivity was present in each mixture The total volume of each culture was then adjusted to 3 ml using Hanks solution and the cultures were incubated at 37 °C for 90 min Each culture contained 3 0–20 5×10^{6} nucleated marrow cells The uptake of ^{57}Co vitamin B_{12} by the bone marrow cells and the effect of various chemicals on vitamin B_{12} uptake were determined as described previously [25] Each sample was counted so that the sample count corrected for the background count had a CV<5% except for the TCIII mediated uptake in cases 6 and 7 where the CV was 30%

Results

The results are shown in table I It can be seen that bone marrow cells took up vitamin B_{12} from all 3 transcobalamins In each instance more counts of ^{57}Co B_{12} were transferred by TCII than by the other two binders, TCI mediated 31–72% (mean 48%) and TCIII mediated 5–83% (mean 41%) of the uptake mediated by TCII

The characteristics of vitamin B_{12} uptake from the 3 binders were similar The uptake was inhibited by 10^{-2} M Na_2EDTA (table II) This inhibition was partially or completely corrected by the addition of 1 5×10^{-2} M calcium chloride Uptake was also inhibited by 5×10^{-2} M sodium cyanide and 5×10^{-3} M 2 iodocetamide (table II)

Table I Transfer of radioactive vitamin B_{12} from the 3 transcobalamins to human bone marrow cells *in vitro*

Source of marrow (case)	Source of transcobalamin (subject)	Nucleated marrow cells in culture × 10⁶	Radioactivity added cpm	Uptake of ^{57}Co-B_{12} cpm TCII	TCI	TCIII
1	A	20 5	6 660	67	32	12
2	B	9 8	10,250	54	17	45
3	B	12.0	10 100	107	[1]	85
4	B	6 5	9,200	135	[1]	59
5	C	6 0	5,380	116	54	56
6	D	3 0	5 440	45	18	3
7	E	4 0	8 130	60	43	3

[1] *Not tested*

Table II Effect of various inhibitors on the transcobalamin mediated uptake of ^{57}Co-B_{12} by human bone marrow cells

B_{12}-binder	Average inhibition of uptake % 10^{-3} M Na_2EDTA	5×10^{-3} M NaCN	5×10^{-3} M iodoacetamide
TCI	50 7	61 4	41 7
TCII	53 7	29 9	27 2
TCIII	83 6	31.5	24 5

Discussion

The functions of the 3 transcobalamins have not yet been fully elucidated As patients with an apparently complete deficiency of TCII suffer from a severe megaloblastic anaemia [14] and as patients with a partial deficiency of TCI seem to have normal erythropoiesis [5] it is generally believed that the B_{12} binder involved in the transfer of vitamin B_{12} to tissue cells is TCII and not TCI This view is also consistent with the observation that vitamin B_{12} bound to TCI and TCIII clears more slowly from the circulation than vitamin B_{12} bound to TCII [8] However

the above observations do not completely eliminate the possibility that TCI and TCIII participate in the transfer of some vitamin B_{12} to tissue cells, particularly as the cases of megaloblastic anaemia associated with TCII deficiency were also complicated by malabsorption of vitamin B_{12} [14] and by some atypical biochemical features [19].

The few studies of the ability of the 3 transcobalamins to transfer vitamin B_{12} to tissue cells *in vitro* have provided somewhat conflicting results. Thus, FINKLER and HALL [9] showed that vitamin B_{12} bound to TCII was taken up by monolayers of HeLa cells and by primary cultures of human kidney and amnion cells. These cell types did not take up vitamin B_{12} bound to TCI. By contrast, RETIEF *et al.* [18] found that both TCI and TCII were capable of transferring vitamin B_{12} to reticulocyte-rich human red cells. The results of the present study show that TCI, TCII and TCIII are all capable of transferring vitamin B_{12} to marrow cells (table I), but that TCII transferred more counts than TCI or TCIII. These results, however, do not necessarily imply that TCII is relatively more important than TCI or TCIII. In fact, as the majority of the vitamin B_{12} in normal serum is bound to TCI [3], it is quite possible that marrow cells take up most of thier vitamin B_{12} from this binder *in vivo*.

The uptake of vitamin B_{12} from all 3 transcobalamins was inhibited by 10[illegible] M Na_2EDTA (table II). This inhibition was corrected by the addition of $1.5\times10^{-}$[illegible] M calcium chloride, indicating that uptake was dependent on the presence of calcium ions. Uptake of vitamin B_{12} from all 3 transcobalamins was also inhibited by sodium cyanide and 2 iodoacetamide (table II), indicating its dependence on cellular respiration and free sulphydryl groups. Thus the uptake of vitamin B_{12} from all 3 serum binders is mediated via a specific calcium dependent, iodoacetamide sensitive transport process and differs from the serum independent uptake of free vitamin B_{12} which has previously been shown [25] to be unrelated to the presence of calcium ions or free sulphydryl groups.

In a recent study, MEYER *et al.* [16] measured the uptake of vitamin B_{12} by PHA-stimulated human lymphocytes, found that the uptake mediated by TCI or TCIII was quantitatively similar to the uptake of unbound vitamin B_{12} and concluded that TCI and TCIII played no role in the transfer of vitamin B_{12} to tissue cells. However, as the present data on human bone marrow cells indicate that the mechanism of uptake of vitamin B_{12} from TCI and TCIII is different from that of the uptake of unbound vitamin B_{12} the results of MEYER *et al.* [16] may have to be

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Urine Porphyrins and Their Precursors in Homozygous β-Thalassaemia

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Key Words Haem biosynthesis Porphyrin metabolism Thalassaemia Urine porphyrins

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The fundamental defect in the thalassaemias resides in the protein moiety of haemoglobin Nevertheless haem biosynthesis is also disturbed [1–4 12, 13] More recently, a considerable increase of free protoporphyrin [6] and of the activity of haem enzyme δ-aminolaevulinic acid dehydratase [7] has been found in thalassaemic erythrocytes Data on the excretion pattern of urine prophyrin in thalassaemic syndromes are limited and the results rather conflicting The present paper describes investigations on the urinary excretion of porphyrins and porphyrin precursors in a large series of patients with homozygous β thalassaemia

Material and Methods

The material consisted of urine samples from 68 patients aged 2–25 years with homozygous β thalassaemia Urine specimens from 30 normal subjects matched for age and sex served as controls The first morning urine was analyzed. Urine porphyrins were measured according to Rimington [10] For the quantitative determination of δ-aminolaevulinic acid (ALA) and porphobilinogen (PBG) the technique of Mauzerall and Granick [8] was followed

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Table I Urine porphyrins and porphyrin precursors in thalassaemic patients and normal subjects

	ALA, mg/l	PBG, mg/l	CP, µg/l	UP, µg/l
Thalassaemia	0 36−3 04 (1 93±0 61)	1 275−9 000 (5 682±2.050)	53 03−369 95 (167 78±82 75)	2.08−18 72 (9 44±4 06)
Normal subjects	0 80−2 88 (1 78±0 56)	0 375−4 500 (2 486±1 117)	22 09−108 27 (68 47±25 95)	1 04−16 64 (4 84±3 33)

The numbers in parentheses represent the mean values ± standard deviation of the means ALA = δ Aminolaevulinic acid PBG = porphobilinogen CP = coproporphyrin UP = uroporphyrin

Results

The results are summarized in table I Many thalassaemic patients exhibit urine PBG and coproporphyrin (CP) values within the normal range, but the mean values of the thalassaemic group is significantly higher than the corresponding values of the control group ($p<0\ 0005$) ALA and uroporphyrin excretion was similar in both groups

Discussion

Attempts of previous workers to establish a porphyrin excretion pattern in urine of thalassaemic patients have not given definite results This can be attributed to the relatively small number of investigated cases Thus, SCHWARZ-TIENE *et al* [11] have found a normal or raised urine excretion of CP in 10 cases of thalassaemia major, its pattern was found to be normal in the parents of these patients PRATO and MAZZA [9] found an only very slightly increased excretion of CP in urine in 10 patients with thalassaemia major and 8 subjects with the minor form HEILMEYER *et al* [5] found increased CP and ALA excretion in urine in 14 out of 15 cases of thalassaemia minor The PBG excretion ranged between very low and high values BANNERMAN *et al* [3] found the total porphyrin excretion in the 24-hour urine collection to lie between 1,000 and 2,000 µg in a patient with β-thalassaemia intermedia

In the present series, a notable, even remarkable increase in urine excretion of CP and PBG was found The uroporphyrin excretion ranged

mostly around the upper limit of normal ALA excretion was similar in the two groups In conclusion, in spite of the accumulated data a definite porphyrin and porphyrin precursor excretion pattern in urine of thalassaemic patients cannot be established Nevertheless the present study showed that the majority of β thalassaemics do excrete in urine a large amount of CP and PBG

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L'agrégation des plaquettes à l'ADP: Etude par diffusion de la lumière[1]

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Key Words. ADP · Light absorption · Light scattering · Nephelometry · Platelet aggregation · Platelet shape · Thrombocytes

Abstract. The absorption and the ADP induced platelet aggregation have been studied by the technique of light scattering. The results show, that the platelets have no specific absorption and that the aggregation test measures predominantly the diminution of free platelets. The determination of platelet kinetics and shape changes is not specific.

L'etude de l'agrégation plaquettaire est généralement approchée, d[e]puis plusieurs années, à l'aide de la technique photométrique propos[ée] par Born [1]. Cette méthode consiste à étudier les variations de turbi[-]dité d'un plasma riche en plaquettes (PRP) au cours de l'agrégation. Une modification récente proposée par Michal [10] a permis d'étudier les variations de lumière diffusée à 90°.

Partant d'un travail précédent [12] où nous avions envisagé le calcul du volume diffusant des plaquettes, notre objectif est de dégager les différents paramètres qui interviennent dans la méthode néphélométrique classique, afin de préciser les phénomènes mesurés et la signification qu'il faut attribuer aux courbes enregistrées. C'est pourquoi nous avons étudié d'une part, l'absorption propre des plaquettes comparée au plasma et, d'autre part, les variations d'intensité lumineuse diffusée à un angle donné, ou transmises par un PRP au cours de l'agrégation à l'ADP.

[1] Ce travail a été réalisé avec l'aide de la DRME (Section Biologie). Contrat no 74 34 202 00 480 75 01.

Methode

Rappels théoriques concernant la néphélométrie La turbidité spécifique d une solution est definie comme le logarithme du rapport des intensités lumineuses inci dentes et transmises rapporte à l unite de longueur de solution traversée Cette turbidite a principalement deux causes 1) l absorption propre (et generalement selective) des molecules ou particules en suspension (solutions colorees) 2) la dis s pation de lumière par effets de diffusion ou de fluorescence dans d autres direc tions que celle de la lumière incidente (solutions troubles)

Les deux phénomènes interviennent simultanément mais dans la pratique on distingue deux cas extremes 1) les particules ou molécules presentent une absorption propre notable et sont suffisamment petites pour que la lumière diffu ee soit négligeable La loi de Beer Lambert s applique alors et la turbid té est proportion nelle à la concentration 2) les particules ne presentent pas d absorption propre importante et sont d assez grande dimension Seul intervient dans ce cas l effet du à la diffusion en effet la somme de toute l intensité diffusée à angle non nul et de l intensité lumineuse transmise est égale en l absence de fluorescence à l in tensité incidente

Il n y a donc pas de différence fondamentale entre l étude de la turbid te ou de la diffusion et les calculs presentes par DEBYE [5] et DOTY et STEINER [6] portant sur l étude de la turbidite de solutions de macromolecules montrent que les me sures de turbidité permettent d atteindre les memes parametres que les études de diffusion et que les equations sont similaires

Matériel Nous avons utilisé un photogoniodiffusomètre Fica 50 qui a été decrit par ailleurs [12] Les mesures d absorption ont eté faites à l aide d un spec trophotomètre Beckmann entre 0 3 et 0 7 µm

Le sang nécessaire à la préparation des PRP a été recueilli en verrerie siliconee sur citrate ou sur EDTA Les PRP etaient prepares par centrifugation lente du sang total à 18 °C Le plasma pauvre en plaquettes (PPP) était obtenu par centrifugation rapide du PRP

L ADP provena t des laboratoires Sigma

Méthode Notre étude experimentale comporte trois parties 1) l étude de l absorption propre du PRP et des plaquettes 2) l etude de l agrégation à l ADP et 3) l influence de l ADP sur le PRP recueilli sur EDTA

Les mesures ont éte effectuées à 37 °C L ADP était ajouté à la pipette de fa çon à éviter les turbulences

L ADP était prepare en solution aqueuse isotonique à une concentration telle que l addition d ADP represente 2,5‰ en volume du volume total du PRP placé dans la cellule Ceci afin de négliger l effet de la dilution Les doses utilisées ont var e de 0 5 à 2 µg ml

Résultats

Etude de l absorption propre du PRP citraté Les courbes d absorption pour le PRP et le PPP sont résumees sur la figure 1 (comparee au

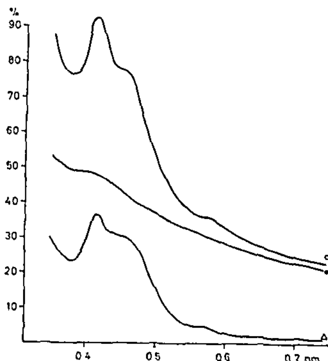


Fig 1 Courbes d absorption (%) du PRP et du PPP en fonction des longueurs d ondes (nm) ○ = PRP avec comme référence l eau, △ = PRP avec comme référence le PPP, ● PPP avec comme référence l eau

solvant, c'est-à-dire a l'eau) Il apparaît que le PPP présente une absorption propre importante vers 0,45 μm Par conséquent, le PRP (qui est une suspension de plaquettes dans le PPP) présente également un pic d'absorption à cette longueur d'onde Par contre, en prenant comme reference le PPP, le PRP ne présente pas de pic d'absorption propre, la courbe est continue et assimilable a une courbe en $1/\lambda^2$ (λ=longueur d'onde de la lumière) On peut donc penser que les plaquettes seules ne présentent pas d'absorption propre notable aux longueurs d'onde utilisées Par ailleurs, comme l'a montré KASTLER [8], l'absorption apparente (due à la diffusion) d'une solution de particules de dimensions comparables à la longueur d'onde de la lumière utilisée variait en $1/\lambda^2$ Par conséquent, la turbidité du PRP provient de la diffusion des plaquettes et non du phénomène d'absorption

Etude de l agrégation (PRP citrate) Nous avons étudié l'agrégation des plaquettes par l'ADP à différentes doses 0,5, 1 et 2 μg/ml et à dif-

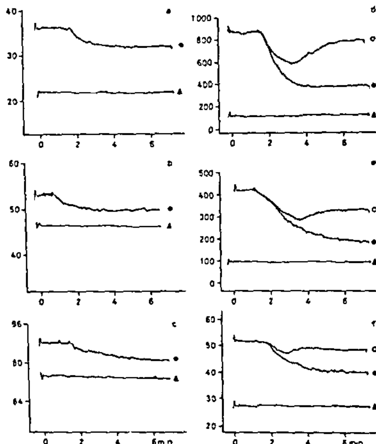


Fig 2 Courbes de diffusion de la lumière au cours de l agrégation des plaquettes. Les courbes représentent les variations au cours du temps (min) de l intensité lumineuse diffusée (valeurs analogiques) au cours de l'agrégation du PRP (● = 2 µg ml, ○ = 0,5 µg ml d ADP) et du PPP (▲) Angles d'observation a = 90°, b = 120°, c = 142,5°, d = 30°, e = 45° f = 75°

ferents angles Les courbes obtenues sont reproductibles quant à leur aspect, bien que les intensites lumineuses diffusées ou transmises varient de façon sensible d un PRP à l'autre La figure 2 donne un échantillon représentatif des courbes obtenues a differents angles pour un PRP citraté et pour deux doses d ADP (0,5 et 2 µg/ml) En ce qui concerne l interprétation, il faut remarquer que les agregats sont de grande dimen-

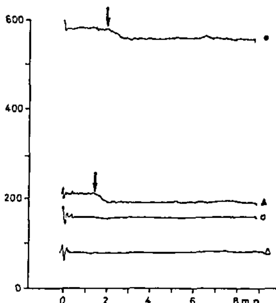


Fig 3 Influence de 2 µg/ml d ADP sur la diffus on de la lumiere par des plaquettes prelevées sur EDTA ● = PRP observe à 60° ▲ = PRP observé à 90° ○ = PPP observé à 60° △ = PPP observe à 60°

sion par rapport aux autres elements et diffusent tres peu de lumiere du fait des interferences internes De plus, ils sont en concentration tres faible par rapport a celle des plaquettes Par suite, ils interviennent tres peu dans la turbidite de la solution qui provient essentiellement des plaquettes libres donc les courbes enregistrees traduisent essentiellement la diminution du nombre des plaquettes libres dans le milieu au cours de l agregation Dans la premiere phase, ces courbes sont assimilables en premiere approximation a des exponentielles decroissantes Ceci est du en grande partie, au fait que la diffusion varie exponentiellement avec la concentration en plaquettes dans le domaine de concentration etudie [12]

Influence de l ADP sur la diffusion des plaquettes prelevees sur EDTA Le PRP obtenu à partir de sang recueilli sur EDTA n'agrege pas a l ADP Toutefois l addition d ADP a une dose finale de 2 µg/ml produit une legere diminution de l intensite lumineuse diffusée (fig 3) Il faut egalement noter que la diffusion par le PRP citrate est differente de celle du PRP preleve sur EDTA L effet de diffusion multiple semble plus important dans le cas du PRP citrate

Discussion

Agregation Le premier point que notre étude permet de preciser est que, comme l avait remarque HUGUES [7] les courbes dites «d agrégation» ne traduisent en fait que la diminution du nombre de plaquettes libres dans le milieu au cours de l agrégation Par suite, le test nephélometrique renseigne essentiellement sur le nombre de plaquettes mises en jeu dans la reaction d agregation, et non sur la façon dont se forment les agregats Il est alors interessant de noter que le modele mathematique propose par CRONBERG [4] pour rendre compte des courbes experimentales d agregation suppose que les particules en suspension sont parfaitement absorbantes (corps noir) Il ne tient pas compte de la diffusion bien que cet effet soit largement predominant

Certains auteurs, comme SKOZA *et al* [11] ont fait des mesures de la pente initiale des courbes d agregation pour aborder la cinetique de cette reaction Une telle demarche n est pas exacte, etant donnee la forme exponentielle des courbes de variation de l intensite lumineuse diffusee en fonction de la concentration En effet si la concentration en plaquettes varie lineairement avec le temps la courbe enregistree de l'intensite diffusée ou transmise sera exponentielle Et, de toutes façons, de telles mesures ne renseignent que sur la vitesse de disparition des plaquettes libres

Par consequent la cinétique d agregation n est pas traduite directement par la courbe néphélometrique ou de diffusion puisque le phénomene enregistre n est pas proportionnel a la réaction d agregation Il est d ailleurs delicat de deduire une cinetique des courbes enregistrees, car la variation d intensite lumineuse transmise ou diffusee est tres rapide au depart et l erreur de mesure se repercute exponentiellement sur la valeur de al vitesse initiale de disparition des plaquettes que l on voudrait calculer

Probleme du «changement de forme» Un certain nombre d auteurs ont observe dans les premieres secondes de l agregation, une legère variation de l intensite diffusee ou transmise qu ils attribuent, en s'appuyant sur l etude de MACMILLAN et OLIVER [9] à un changement de forme des plaquettes En augmentant la sensibilite de l appareil de façon a ce qu une variation de 10% de l intensite transmise ou diffusee remplisse l echelle complète de l enregistreur et en travaillant sur PRP EDTA, ils ont ainsi etudie les variations d intensite lumineuse transmises lorsqu on ajoute l ADP

Dans le cas du PRP citrate, nos mesures n'ont pas mis en evidence de tels phenomenes Dans le cas du PRP-EDTA, par contre, nous avons note une legere variation de l intensite lumineuse diffusee et transmise Mais il semble delicat d attribuer une signification precise a de telles variations pour deux raisons essentielles 1) une modification de la forme des particules diffusantes modifie l indicatrice de diffusion et les interferences internes Pour atteindre les variations de volume ou de forme, il faudrait donc effectuer des mesures a differents angles en utilisant par exemple, la methode de BRYANT *et al* [3], 2) tant en ce qui concerne la nephelometrie que la diffusion, la forme des particules n intervient que tres indirectement dans ces phenomenes, d autant que la relative polydispersite des plaquettes (polydispersite de forme et de volume) et l'effet de diffusion multiple ne permettent pas d interpreter dans ce sens des variations, d ailleurs tres faibles, d intensite lumineuse ou diffusee

Par ailleurs, les fluctuations en concentration en plaquettes au sein du PRP même agite a 1000 tpm ainsi que la forte concentration en proteines du PPP, font varier la ligne de base de diffusion ou de transmission du PRP autour de sa valeur moyenne et rendent les mesures peu precises

Par consequent, l imprecision relative des mesures et le grand nombre de parametres qui affectent les phenomenes ne permettent pas d'attribuer a un changement de forme des plaquettes les variations d intensite lumineuse diffusee ou transmise par un PRP, observees par BORN [2] au debut de l'agregation à l ADP D autres mesures et d autres techniques sont necessaires pour mettre en evidence un tel changement de forme eventuel

Conclusion

Les mesures de diffusion et nephelometriques permettent de preciser la signification des courbes d agregation qui traduisent la disparition progressive d une partie des plaquettes libres pour former des agregats C est ainsi que le test classique nephelometrique renseigne essentiellement sur le nombre de plaquettes libres mises en jeu au cours de l agregation mais ne permet pas l etude de la formation ou de la structure des agregats Il n'est pas possible de tenter d aborder par cette methode les problemes de cinetique ou de changements de forme eventuels de cet element a la phase initiale de l agregation

Resumé

Les auteurs etudient l absorption propre des plaquettes et l agregation plaquettaire à l ADP par la technique de diffusion de la lumière Les résultats montrent que les plaquettes ne presentent pas d absorption propre et que le test d agrégation mesure essentiellement la diminution du nombre de plaquettes libres Les mesures de cinetique d agregation ou de changements de forme ne sont pas specifiques

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The Sézary Syndrome Cell: Surface Ultrastructural Characteristics

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Key Words Leukemia Mycosis fungoides Scanning electron microscopy Sézary syndrome Surface microvilli T lymphocytes

Abstract Peripheral blood mononuclear cells from a patient with Sézary syndrome which lacked E rosette forming ability and surface immunoglobulins and which displayed a markedly depressed response to a variety of mitogens, were studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) on 3 occasions The first peripheral blood sample (smooth) differed significantly from two later samples (moderate numbers of microvilli) when surface characteristics were examined by SEM these differences were confirmed by TEM The Sezary syndrome cells in this patient may be related to a T lymphocyte which has lost certain surface markers and mitogen response characteristics through a process of de differentiation

Pruritic erythroderma, lymphadenopathy, and the presence in the peripheral blood of large numbers of abnormal mononuclear leucocytes characterize the Sezary syndrome (SS), a variant of mycosis fungoides (MF) Their highly convoluted nuclei and scanty basophilic cytoplasm distinguish Sezary cells from normal lymphocytes or monocytes Wheth-

er Sezary cells belong to the lymphocyte or monocyte cell line has been much disputed in the past Recent studies using transmission electron microscopy (TEM) and immunologic surface marker analyses stronely support their lymphoid nature [4–6, 12, 20]

A recent report of abnormal surface properties and mitogen responsiveness in 5 cases of SS did not permit the identification of the SS cells as either B (thymus independent) or T (thymus-dependent) lymphocytes [3], although it was concluded earlier that SS cells are of T cell origin [4, 5, 22]

Surface ultrastructural studies of peripheral blood leucocytes have demonstrated that lymphocytes can be differentiated from monocytes by this method [8, 15 16] Human lymphoid cells may be divided into two groups on the basis of their surface morphology B lymphocytes and T lymphocytes Most B lymphocytes are more villous than their thymus-derived counterparts T lymphocytes display smaller numbers of microvilli and are smoother There are intermediate forms however, which are difficult to classify [10 13–16] Monocytes from normal peripheral blood and from patients with monoblastic leukemic and nonspecific monocytosis have recently been studied by scanning electron microscopy (SEM) [15 16] The predominant surface features are broad based undulating ruffles with no microvilli

We report here on the use of SEM (on 3 different occasions) in conjunction with TEM for a determination of the surface ultrastructural characteristics of the circulating cells of a patient with an unusually high proportion of Sezary cells (80% or greater) The results are correlated with lymphoid cell surface markers mitogen responsivenesss and with the patient s clinical course

Case Report

M N was a 60-year-old white female who at age 58 (July 1972) had com plained chiefly of an erythematous leg rash which persisted for about 1 year and which, on skin biopsy was interpreted as *mycosis fungoides The findings of* a staging laparotomy (September 1972) were entirely negative [21] A summary of the patient s clinical course and blood counts is presented in table I

The patient expired January 14 1974 18 months after the initial diagnosis was made Autopsy revealed MF of the skin liver bone marrow and axillary and per iaortic lymph nodes A subarachnoid hemorrhage of *moderate size diffuse small* bowel hemorrhage and acute peritonitis were also noted

cocytes. The number of abnormal cells in the mononuclear population was estimated in Giemsa stained smears. That these estimates are accurate was confirmed previously by TEM [20]. The cells were washed 3 times in RPMI culture medium and were collected by filtration onto silver membranes of 0.2 μm porosity [22]. The membrane layered cells were fixed immediately for at least 1 h with 2.5% glutaraldehyde in Millonig's buffer, rinsed twice with buffer, postfixed in 1% osmium tetroxide for 1 h at 4 C, rinsed twice with buffer, dehydrated in a graded series of alcohol and amyl acetate for 5 min each, and then dried in carbon dioxide according to the critical point drying method described by ANDERSON [2]. Portions of the membrane were attached to stubs, using metal clips [9], and coated with gold palladium on a rotatory stage. The specimens were then stored under vacuum until examination. There was no variation in preparation time, medium, room or solution temperature, or filtration collection procedures from one sample to another.

The first two preparations for TEM were prepared from buffy coats, and the third was prepared after Ficoll Hypaque separation. Routine procedures for embedding and staining were used [20].

The following procedures on the mononuclear cells of the patient's peripheral blood had already been carried out on 4 occasions at 4 to 6-week intervals and were not repeated at the time of ultrastructural analysis: immunofluorescence studies for surface bound immunoglobulins, E rosette forming ability, and mitogen response studies with pokeweed mitogen, concanavalin A, phytohemagglutinin, and anti thymocyte serum [3].

Results

Figure 1 shows two Sezary cells examined by TEM from the sample of June 1973, with characteristic highly convoluted nuclei. The cytoplasmic borders contained no projections of any type. The specimen of August 1973, also examined by TEM, was similar in appearance. On December 8, 1973, SEM (fig. 2) demonstrated that most of the cells possessed a smooth spherical surface with only rare microvilli. An occasional cell had an intermediate number (20–60) of microvilli per exposed surface area (fig. 2, lower right), and an occasional monocyte with broad based undulating ruffles was intermixed (fig. 3). A control light microscopy preparation made after Ficoll Hypaque separation revealed only mononuclear cells and no red blood cells in the cell population used for SEM.

On January 5 and 8, 1974, SEM (fig. 4) revealed rare smooth cells. Most of the cells had a spherical shape with moderate numbers of microvilli (20–60) per exposed surface area. There were some villous cells, with 100–200 microvilli per exposed surface, and an occasional red

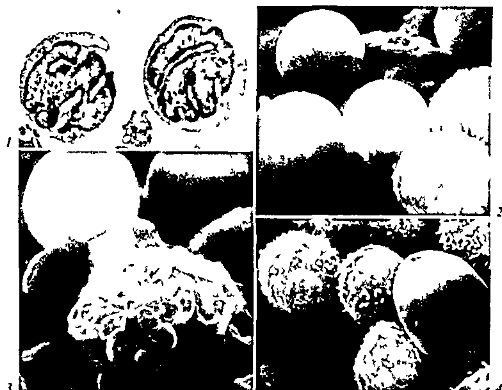


Fig 1 Two cells from June 1973 examined by TEM The nuclei are highly convoluted The cytoplasmic borders demonstrate no projections of any type × 5 250

Fig 2 Smooth spherical cells (left) are the predominant cell type in the first SEM sample (December 8 1973) The cell at bottom right has a moderate number of microvilli whereas the cell above it shows a few scattered microvilli × 4 725

Fig 3 A typical monocyte with broad based ruffled membranes surrounded by smooth spherical cells in the first SEM sample (December 8 1973) × 6 150

Fig 4 Typical area from the third SEM sample (January 8 1974) A characteristic red blood cell is seen with the central concavity at the lower right The remaining cells are spherical and range from moderately to markedly villous 3,375

blood cell Study by TEM on January 8 1974 (simultaneous with SEM of the same day) showed characteristic convoluted nuclei however the cytoplasmic borders of many cells demonstrated moderate numbers of microvillous projections (fig 5)

Supplementary studies performed at 4 different times showed no differences the proportion of lymphoid cells capable of forming E rosettes

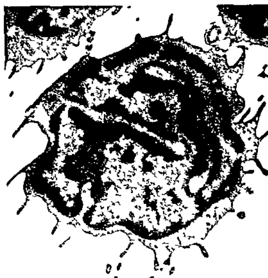


Fig 5 One cell from January 8 1974 examined by TEM The nucleus is highly convoluted The cytoplasmic borders contain moderate numbers of microvilli × 13 000

remained constant (3, 5, 5, and 7%) and the percentage of cells bearing immunoglobulin on their surface remained constant (<3% on all 4 occasions) In general, the response of the lymphoid cell population to all 4 mitogens was markedly depressed [3]

Discussion

The similarity of surface projections demonstrated by both SEM and TEM *in the same sample (January 8, 1974) suggests that the methods* can be freely interchanged when cytoplasmic projections are analyzed The SEM technique allows depth of-field viewing of a cell hemisurface, whereas the TEM method makes possible an exact correlation of cell *identity (i e , nuclear and cytoplasmic characteristics) with* surface projections

The striking difference in surface morphology between the groups of smooth and moderately villous samples cannot be explained by effects

of either the chemotherapy or the antibiotics (table I) Preparation artifacts resulting in excessive smoothness are a possibility, but the presence of normal-appearing ruffled monocytes in the first SEM sample (fig 3) and of cells with a few microvilli adjacent to smooth cells (fig 2) makes this unlikely Recently, LIN *et al* [11] described a temperature-induced variation in the surface topography Changes occurred if the cells were chilled or heated prior to fixation Our preparations were processed at room temperature before the addition of glutaraldehyde PORTER *et al* [18] described cell-cycle-dependent surface changes in ovary cells of the Chinese hamster Microvilli tended to decrease during the S phase, and the cells became relatively smooth as they spread over the substrate Variations in the surface morphology of cells in a single sample might be explained by cell cycle differences, but the similarity of the entire sample of Sezary cells in the several examinations would have to presuppose synchronization of the entire circulating cell population, which seems unlikely Virus-transformed cells in culture have been shown to be different from nontransformed cells of the identical type and display increased numbers and types of surface projections [19]

The absence of surface immunoglobulins, E rosette formation, and mitogen responsiveness in the patient's lymphoid cell population might lead one to expect the cells to have less surface differentiation than normal lymphocytes The occurrence of a population of smooth cells, as in the early samples, is consistent with this hypothesis POLLIACK *et al* [14, 16, 17] have suggested that cells at an earlier stage of differentiation tend to show smoother surfaces than more mature cells Cells from patients with acute monoblastic leukemia have fewer ruffles than normal monocytes [8]

The presence of moderate numbers of surface microvilli in subsequent samples, taken during the terminal phase of the disease, might represent the response of the SS cell to extreme stress (i e, peritonitis and disseminated intravascular coagulation) Alternatively, variation in surface features [17] and loss of surface immunoglobulin [1] with progression of the disease has been noted in the leukemic lymphocytes of chronic lymphocytic leukemia, and the changes in this SS case could be explained by progressive disease

In two recent reports on the examination of Sezary cells by TEM, only smooth cells were found [4, 20] A third report contains two figures, with low-power magnification, of Sezary cells from each of two patients The cells from one are smooth, whereas those from the other pa-

tient have what appears to be an intermediate number of cytoplasmic projections [22] A study of preferential cutaneous infiltration by neoplastic thymus-derived lymphocytes showed a circulating Sezary cell from each of two patients One cell was smooth while the other was very villous [7] The smooth surface on a spherical cell, as revealed by SEM examination in our case prior to the terminal phase, is consistent with a T lymphocyte origin [15, 16] However, the TEM reports of patients with Sezary cells with intermediate to large numbers of cytoplasmic projections [7, 22] and the conversion, in our case of a population of smooth cells to one with intermediate numbers of microvilli require further investigation

Acknowledgment This project was supported in part by United States Public Health Service Training Grant No 5 T01 AM-05623-05 Grant CA 05183 and Grant CA 8970 06 from the National Institutes of Health Bethesda Md., and the Leukemia Research Foundation Inc Acknowledgment to Aaron Polliack, MD Department of Hematology Hadassah University Hospital Jerusalem (Israel) for his careful review and constructive advice

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Burkitt's Lymphoma Presenting as Acute Leukaemia

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Key Words Burkitt s tumour Epstein Barr virus Leukaemia in Burkitt s lymphoma Methylgreen pyronine

Abstract A Nigerian male child presented with extensive superficial lymphadenopathy, a bleeding diathesis, and numerous tumour cells in blood and bone marrow Further characterization of the tumour cells confirmed the diagnosis of Burkitt s lymphoma The main unusual features shown by this patient are discussed

Burkitt's lymphoma is the commonest childhood tumour in Africa, accounting for 53 4% of all malignant tumours and leukaemia seen in children in Ibadan, Western Nigeria [8] Leukaemic features are rare in Burkitt's lymphoma and, to the best of our knowledge, the presence of Burkitt's lymphoma cells in large quantities in the peripheral circulation has only once been reported [9] The present communication concerns another case of Burkitt's lymphoma presenting as acute leukaemia

Case Report

K S (UCH No 321382) an 8 year old Nigerian male child, presented at the University College Hospital Ibadan on August 20, 1973 The complaints were swelling of both cheeks for 2 weeks, bleeding from the gums for 1 week, blood in the stools for 5 days, and protrusion of both eyes for 5 days There was no previous *ill health*

Physical examination revealed pallor of mucous membranes, swollen and bleeding gums, multiple purpuric spots and right subconjunctival haemorrhage There was marked facial swelling with bilateral proptosis The superficial lymph nodes in the submental cervical axillary and inguinal regions were enlarged and discrete

but non tender The liver was 7 cm enlarged and the spleen 8 cm Both organs were firm and non tender

Packed cell volume 15%, haemoglobin 4 5 g/100 ml, white cell count 59,700/μl platelet count less than 10,000/μl The blood film showed numerous blast cells each measuring 10–15 μm in diameter with multiple vacuolations in the cytoplasm and 2–3 nucleoli in each nucleous (fig 1) Marrow aspiration from the left iliac crest showed a very cellular marrow appearance with complete bone marrow replacement by Burkitt's lymphoma cells morphologically identical to those found in the peripheral film (fig 2) Methylgreen pyronine staining of bone marrow aspirate revealed intense pyroninophilia of the cytoplasm of all the blast cells, a feature which is characteristic of Burkitt s lymphoma [2] Phase contrast cytology of peripheral blood cells showed blast cells with dark scanty cytoplasm containing conspicuous lipid granules

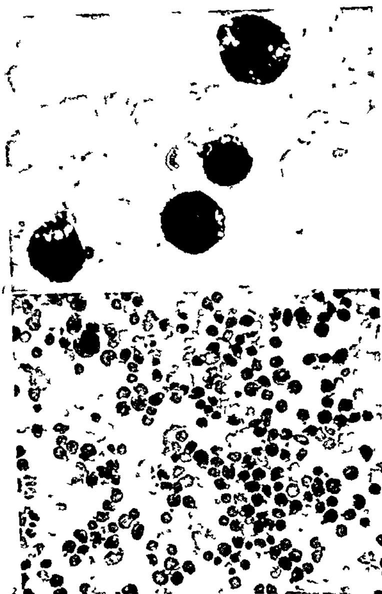
The patient was transfused with 500 ml of fresh whole blood and with 2 units of platelets In addition intravenous vincristine 1 5 mg/m², intravenous cyclophosphamide 500 mg/m² and oral prednisolone 40 mg/m²/day were administered However, he deteriorated very rapidly and died on the fourth day of admission

Autopsy confirmed the diagnosis of Burkitt s lymphoma There was involvement of the jaws, orbits and the long bones The liver, spleen, intestines, and the lymph glands in the cervical axillary, inguinal and retroperitoneal areas were all diffusely infiltrated with Burkitt s lymphoma cells

Discussion

The present case of Burkitt's lymphoma is noteworthy because of the prominence of clinical and haematological features usually associated with acute leukaemia and the extensive involvement of the superficial lymph nodes Various authors have stressed the rarity of leukaemic manifestations in Burkitt's lymphoma [4, 10] However, O'CONOR and DAVIES [6] have reported evidence of leukaemia in the peripheral blood as a terminal event in one patient, and CLIFT *et al* [3] also reported four patients with Burkitt's tumour who developed bone marrow infiltration with tumour cells terminally Only one of these patients had a substantial number of malignant cells in the peripheral blood, and this was following chemotherapy and surgical excision of the tumour It has been suggested that chemotherapy and surgical excision might have caused the spread of tumour into the blood stream in that particular patient In contrast, tumour cells were found in the bone marrow and peripheral blood of our patient on admission, prior to any chemotherapy or surgical procedures

Extensive superficial lymph node involvement is rare in Burkitt's lymphoma [1, 4] Even when the peripheral lymph nodes are swolen, histolo-



F g 1 Burk t s tumour cells n peripheral blood f lm The cells measure 10–15 m n d ame er and have numerous cytoplasm c vacuolat ons. Le shman s sta n 1 000

F g 2 Il ac marrow asp ra e the marrow is crowded w h tumour cells mor pholog cally ident cal to those n the per pheral blood Le hman s sta n × 270

gy usualy shows reactive hyperplasia only, and the enlargements have been regional rather than generalized [2]. However, in our patient, as well as in the report by STEVENS *et al.* [9], the superficial lymphadenopathy was generalized and the lymph nodes showed extensive infiltration with tumour cells.

The rapidly fatal course of the disease in our patient is in accord with the experience of other workers [10]. The very poor prognosis may be due to a deficiency in the immunological competence of these children. African children with Burkitt's tumour have high titres of Epstein-Barr virus (EBV) antibodies [5]. In Ibadan, titres of antibodies to viral capsid antigens of the EBV in children with Burkitt's lymphoma are usually over 1:5,120 [7]. In our patient the level was significantly lower (1:2,560). This might be due to either some impairment of the patient's ability to produce antibodies to this virus, or to mopping up of the antibodies by the Burkitt's tumour cells. Further immunological studies in patients with similar presentations of Burkitt's tumour are required.

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Factor X Friuli Coagulation Disorder

The Demise of the Index Patient

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Key Words Atherosclerosis Bleeding disorders Blood coagulation Factor X Friuli

Abstract The up dated case history of the index patient with the factor X Friuli coagulation disorder is reported The patient, aged 72, died of irreversible shock after progressive jaundice with symptoms of renal, cardiac and hepatic failure The autopsy showed carcinoma of the head of the pancreas, diffuse petecchial hemorrhages, massive adrenal gland hemorrhage and minimal athero arteriosclerotic lesions of all arteries The significance of minimal athero-arteriosclerotic changes in a patient with a congenital coagulation defect is discussed

The index patient with the factor X Friuli coagulation disorder [7, 9] has died in July 1974 at the age of 72 of shock, hepatorenal failure, obstructive jaundice due to a carcinoma of the pancreas and secondary cholangitis

The factor X Friuli disorder was first described by us in 1969–1970 [7–9, 11, 12] So far 11 homozygote patients and several heterozygotes have been reported [10, 21, 24] The condition is transmitted as an autosomal incompletely recessive trait [10, 18, 21, 24] The main feature of the defect consists in the presence of an abnormal factor X which may not or may be activated only very slowly by whole or partial tissue thromboplastin whereas it may still be normally activated by Russell's Viper venom [7–9, 16] It was also demonstrated that prothrombin is normal in Friuli patients both as activity and as antigen [13] and that no inhibitor is present [17, 22] Immunologically, factor X Friuli behaves

as normal factor X both in plasma and is different from coumarin induced abnormal factor X [15, 19, 23]

Case Report

The proposita (Mrs. M P) is a 72 year-old patient whose family and personal history had already been discussed in detail [7, 9] Since that time we have been following her as an outpatient approximately every 2 months. A slight hypertension was detected for the first time in 1969 but no therapy was needed

No major episode of bleeding occurred during all these past years. In 1973 the patient who had been having a small simple goiter for many years, started presenting signs and symptoms consistent with hyperthyroidism. These was atrial fibrillation and signs of left heart failure. A scintiscan revealed that practically all ^{131}I was uptaken by an active node which substituted normal thyroid morphology 24-hour thyroid uptake was 63%, and ^{131}PBI was 0.42% dose/liter of plasma. The patient was treated with 5 mCi of ^{131}I and subsequently kept on thiouracil derivative therapy. No relapse occurred and the cardiac condition improved, but the heart did not revert to sinus rhythm. In march 1974 the patient underwent electrocoagulation for a detachment of the left retina. The procedure was carried out without any bleeding complication after the administration of 3 units of factor X concentrate (Bebulin 500 U). The factor X level at the end of infusion was 55%. In April 1974 the patient started complaining of anorexia, nausea, vague abdominal pain. A few days later jaundice appeared accompanied by the presence of acholic stools. Because of the clinical suspicion of post transfusion hepatitis, the patient was admitted to the Maniago City Hospital in Friuli. On admission the main laboratory findings were as follows RBC 4 900 000 Hb 94%, Ht 45%, azotemia 32 mg%, glycemia 80 mg%, bilirubin tot. 11.4 direct 10.5 indirect 0.9 mg%, SGOT 249 SGPT 104 units

The patient's condition progressively deteriorated in spite of every therapeutic effort. Jaundice deepened and symptoms of liver failure appeared. Oliguria with hyperazotemia and finally anuria developed which led to exitus in a few days. Shock was present during the final hours. The main autopsy findings were: thyroid node, pulmonary stasis, enlargement of the heart, myocarditis, carcinoma of the head of the pancreas encroaching the choledocus and the Vater's papilla with massive biliary stasis, cholangitis, minimal athero-arteriosclerotic changes of all arteries, diffuse petecchial hemorrhages (pleural, pericardial, renal, etc.) massive hemorrhage of adrenal glands.

Discussion

To our knowledge, this is the first autopsy report of an adult patient with either classical factor X deficiency or factor X Friuli disorder. Another patient with the factor X Friuli disorder has died a few years ago

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intravenous administration of analgesics [14]. Since no thromboembolic complications have been described in a patient with either classical factor X or factor X Friuli disorder, it would seem that a factor X defect is able to protect against thrombosis. The experimental induction of thrombous by means of the injection of serum-activated product seems to lend indirect support to these observations [38, 39]. However, data are still too scanty to warrant any conclusion.

Acknowledgment. Mrs. M. P. has been very helpful to us because of her enthusiasm and cooperation. She was always willing to donate us blood for our studies. She has been stuck hundreds of times during these past 8 years and has never complained. Her plasma has been used as reference plasma in almost all our studies and was sent or brought to several other laboratories. Mrs. M. P. was also of paramount importance in convincing the population of the valley where she was born and lived to undergo testing for heterozygote detection and genetic counseling. The authority derived from her age allowed her even a brusque approach towards villagers who were not too willing to undergo testing. Nobody has ever resisted her persuasion. We shall never forget her and each time we will thaw a vial of her plasma we shall remember her dearly.

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at the age of 49 of acute liver failure due to viral hepatitis but no autoptic data are available [38]. The ultimate cause of death was surely an irreversible shock secondary to the bilateral adrenal gland hemorrhage.

The bleeding manifestations presented by Friuli patients are not severe. However, the present observation shows that a congenital clotting defect represents always a threatening condition because of the possibility of massive bleeding in vital organs. The finding of mild or minimal athero- and arteriosclerosis in a patient with a congenital coagulation disorder is of interest. Similar findings have been demonstrated for a patient with Hageman trait [37], and, apparently, in patients with congenital factor VII deficiency [29]. On the contrary, widespread arteriosclerosis has been observed in a patient with hemophilia A [39]. The age of our proposita is such that it is usually accompanied by the presence of moderate but sure athero-arteriosclerotic changes even in areas with low incidence of athero-arteriosclerosis like Friuli. The aorta was smooth and approximately similar to that seen in a 40- to 50-year-old female. Coronary arteries too were almost free of atherosclerosis. Such findings seem of some relevance in view of the fact our patient was also moderately obese and slightly hypertensive, factors which are widely maintained to be often associated with sclerotic changes of the arteries. However, the overall significance of these data is still unclear. The number of patients examined is still too limited to warrant any conclusion or to claim support for DUGUID's [5, 6] theory on the pathogenesis of atherosclerosis.

No thrombus was found in our patient in spite of the fact the stasis secondary to atrial fibrillation and left heart failure was surely present. Thromboembolic manifestations have been described in congenital afibrinogenemia after fibrinogen infusion and or contraceptive medication [4, 31, 33], in factor V deficiency [32], in factor VII deficiency [25–27, 29, 30] and in factor XII deficiency [36]. All these defects seem unable to protect against a thrombus formation. As a matter of fact, the observation that five patients with congenital factor VII deficiency presented thromboembolic manifestations, would seem to suggest the paradox that factor VII deficiency is frequently associated with thrombotic complications. Only about 100 patients with sure or probably factor VII deficiency have in fact been described so far [25]. Myocardial infarctions have been described in a few patients with Hageman trait [27, 31, 39], with hemophilia A [1, 2] or hemophilia B [3, 33]. A superficial phlebitis was described in a hemophilia A patient's and was probably secondary to the

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sur sang total Le chapitre thérapeutique rappelle les connaissances indispensables dans tous les cas à la bonne conduite d un traitement anticoagulant l action des antivitamines K et de l'heparine dans les états thrombophiliques est analysee L'accent est mis sur les traitements preventifs possibles reposant sur l utilisation de l'héparinate de calcium en cures discontinues Enfin les moyens de surveillance de ces traitements sont présentés

Le livre se termine par treize planches en couleurs schématisant la physiologie de la coagulation le mode d action des anticoagulants la physiopathologie des CID et les rapports entre troubles de l hémodynamique et CID Ce livre présente dans l ensemble l intérêt immense de proposer un mode de raisonnement logique sur les états d hypercoagulabilité De ce raisonnement et des hypothèses qui en decoulent se degagent les voies d exploration biologique et les règles thérapeutiques à appliquer

J Y MULLER Paris

A ENGELHARDT und H LOMMEL (Hrsg) **Serumproteine.** Methodische Fortschritte im medizinischen Laboratorium Vol 1 Verlag Chemie Weinheim 1974 230 pp 67 fig., 40 tab , DM 52

Der 230 Seiten umfassende Band ist in die folgenden Kapitel unterteilt 1 Albumine und Globuline 2 spezielle Proteine 3 Paraproteine, 4 Lipoproteine 5 Enzyme und Isoenzyme 6 Antikorper Das erste Kapitel befasst sich mit der elektrophoretischen Auftrennung humanen Serums auf Zelluloseazetatfolien Die Methode wird detailliert beschrieben wobei auch Normwerte und Methodenstreuung diskutiert werden Hinweise auf die klin sche Bedeutung der verschiedenen Eiweissbilder werden nicht gegeben Das zweite Kapitel enthalt Abschnitte über die quantitative Bestimmung einzelner Proteine Das Hauptgewicht wird auf die radiale Immundiffusion gelegt (Mancini Technik) E n Abschnitt behandelt auch die eindimensionale Immundiffusion (Oudin) die wegen zu grosser Ungenauigkeit heute praktisch nicht mehr verwendet wird dafur fehlt d e E [illegible] orterung der Laurell Methode fur die Bestimmung einzelner Proteine wel h [illegible] e als die beste gilt Die zwe dimensionale Immunelektrophorese (Modifikat [illegible] Laurell Technik) welcher ein Aufsatz gewidmet wird gibt zwar spektaku [illegible] hat sich aber in der Praxis nicht bewahrt, da gerade bei quantitativen \ [illegible] ungen eine Identifikation der einzelnen Proteine auf Schwierigkeiten stosst D [illegible] gische Funktion und die Eigenschaften von Praalbumin α_1 Antitrypsin Zar [illegible] asmin α_2 Makroglobulin Haptoglobin Hamopexin und Transferrin werden k [illegible] hes brieben von einigen wird der klinische Wert einer quantitativen Best mm [illegible] iskut ert Zwei weitere Abschnitte befassen sich mit den Möglichkeiten der [illegible] horese in Polyacrylamid Im dritten Kap tel wird besonders auf die Diagnost [illegible] mmunchemischen Methoden und deren Probleme eingegangen Das vierte Kap [illegible] einhaltet die Lipoproteinanalytik In mehreren Abschnitten werden die Metn [illegible] r Analyse der verschiedenen Lipoproteinsammemuster dargelegt Agarosege [illegible] Zelluloseazetatfolienelektrophorese werden ausführlich beschrieben Die klin [illegible] Bedeutung der funf wichtigsten Lipoproteinmuster wird kurz erwahnt Eine Arb [illegible] ist dem Nachweis von LP X gewidmet das in jungerer Zeit fur die Different [illegible] gnose ikterischer Erkrankungen an Bedeutung gewonnen hat Das funfte Kap tel [illegible] deh die Diagnostik der Isoenzy

A LARCAN J F STOLTZ and F STREIFF **La charge electrique des élements figurés du sang** Doin Paris 1974 170 pp FF 138 –

The book contains an excellent review of the basical knowledge the methods for measuring the electrophoretic mobility and the electric charge of normal and abnormal blood cells and their diagnostic implications More than 500 references are given

H R MARTI *Aarau*

C RABY **Coagulations intravasculaires disséminées et localisées** 2e ed Masson Paris 1974 247 pp 45 fig 16 tab 4 planches en couleurs SFr 72 –

La deuxième édition du livre de RABY *coagulations intravasculaires disséminées et localisées* depasse en réalite le cadre des coagulations intravasculaires dissémi nees (CID) pour faire le point des connaissances actuelles et des conceptions de l auteur sur les états d hypercoagulabilite

Le livre est divisé en deux parties la première résume les donnees essentielles concernant l hémostase physiologique et ses modes d exploration Une part impor tante est consacree à la mise en évidence de l equilibre normal existant entre fonc tion coagulante et fonction coagulolytique ainsi qu aux déséquilibres possibles pou vant aboutir soit à un état thrombophilique soit à un état hémorragique Cette pre miere partie s acheve par une étude des facteurs thrombogenes classés en facteurs vasculaires facteurs sanguins et facteurs hemodynamiques et par une classification des etats thrombophiliques divises en hypercoagulabilité chronométrique hypercoa gulabil té structurale et hyperadhesivite plaquettaire

La seconde partie est consacree aux CID proprement d tes Leur évolution biolo gique est divisee en 3 phases une premiere phase d hypercoagulabilité réelle chro nométrique une seconde phase ou existent à la fois une hypercoagulabilité poten tielle persistante (du fait d une activite thromboplastique persistante) et une hyper coagulabilite chronometrique liee à la consommation et une troisième phase où à la coagulopathie de consommation s ajoute une fibrinolyse Les différents facteurs susceptibles d induire une CID sont presentes les relations entre les systèmes hemo statique lytique et des kinines sont decrites ainsi que les principaux types de chocs Les circonstances cliniques dans lesquelles peuvent survenir des syndromes de CID sont étudiees une à une Parmi ces differents tableaux cliniques il convient de noter les hypothèses intéressantes faites sur les mécanismes de l atherosclérose et sur l in tervention possible d une CID au cours de purpura thrombopenique idiopathique ou immuno allergique Une observation intéressante de CID secondaire à une morsure de crotale viridis est rapportée La fin de cette seconde partie est consacrée d une part aux tests biologiques permettant de diagnostiquer une CID ou de dépister un état thromboph l que et d autre part au traitement de ces syndromes Parmi les tests biologiques utilisables l accent est mis sur l intéret de la thromboélastographie

sur sang total Le chapitre thérapeutique rappelle les connaissances indispensables dans tous les cas à la bonne conduite d un traitement anticoagulant, l action des antivitamines K et de l'héparine dans les états thrombophiliques est analysée L accent est mis sur les traitements préventifs possibles reposant sur l utilisation de l'héparinate de calcium en cures discontinues Enfin les moyens de surveillance de ces traitements sont présentés

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vestigations were undertaken Some interim reports have been given [23, 24], but this is the first publication of the results up to 1974

Anti-human antilymphocytic globulin (AHLG) was selected as the immunologic tool to identify lymphoid cells by means of indirect immunofluorescence (IMF) Antilymphocytic globulin (ALG) has been thus demonstrated to fix on canine [12], murine [35] and human [30] lymphocytes With fixed and dried lymphocyte preparations, titres up to 1 256 were obtained [35, 36] Unabsorbed ALG, however, seems quite uncapable of distinguishing T and B lymphocytes from each other its uptake was identical for the two subpopulations in BRAIN and MARSTON's experiments [5], while selective reactions against T or B lymphocytes were obtained only after specific absorbtions, even when apparently pure subpopulations had been used as immunogens [1, 32] Two subpopulations of lymphoid cells in quiescent rat lymph nodes, which grew to three after peak response to *Salmonella typhi* H antigen, were found by using rabbit anti rat thymocyte globulin and estimating IMF by spectrophotofluorimetry [29] Lymphocytes in human tissue sections showed varying binding of AHLG raised against peripheral blood lymphocytes from chronic lymphatic leukaemia (CLL) and from normal individuals [33], the marked cross-reaction of neutrophilic granulocytes was considered as due to a common antigen Finally, the development of an anti-AL serum capable of distinguishing ALL cells from both normal lymphoblasts and other leukaemic cells has been briefly announced most recently [6]

Although some of these studies were performed with fixed lymphocyte preparations [12, 30, 35, 36] and others with fresh suspensions [1, 5, 30], transmitted excitation was used in the great majority

Materials and Methods

The diagnoses of CLL acute myeloblastic leukaemia (AML) and of the acute agranular blastic crisis of chronic myelogenous leukaemia (CML BC) were based on clinical and haematologic criteria including Romanowsky stained smears and cytochemical examinations

Cell preparations Myeloid leukaemic cells from AML and CML BC were obtained by centrifugation of EDTA treated venous blood and recovery of buffy coats The cells were then washed 3 times with 10% fetal calf serum in Hanks' balanced salts solution adjusting pH with Sorensen's buffer 0 066 M between 7 4 and 7 45 Lymphocytes from CLL and lymphoblasts from ALL were separated by centrifugation on a Ficoll Urovison density gradient [16] and washed

3 times as described above Concentrations of 10^6 cells ml were obtained for the final preparation

AHLG Two commercial AHLG preparations were used in a first group of studies most predominantly Behringwerke s horse AHLG raised against peripheral blood lymphocytes from normal donors, but also Searle s rabbit AHLG raised against cultured lymphoblasts A second group of experiments was performed with Behringwerke s refined antisera raised against lymphocytes from various sources tonsil (TO) spleen (SP) lymph node (LN) thymus (TY) thoracic duct (TD) CLL blood (CLL) and cultured lymphoblasts (KL) The $(Fab)_2$ fraction of the latter and of the anti TD AHLG were also furnished by Behringwerke as well as FITC-conjugated anti TD anti CLL, anti-KL and anti mixed (MX) lymphocytes AHLG All preparations were obtained from horses immunized with whole-cell suspensions, except for the anti TD AHLG elicited with whole cells and membrane fractions in separate batches Searle s rabbit AHLG was also raised with membrane fractions of KL. The protein concentration of the antisera ranged from 4 to 7 g% of pure Ig the horse preparations comprised IgG and IgT and at least 90% are 7S immunoglobulins [15]

Myeloid absorption Myeloid adsorbed AHLG was prepared by incubating 2.5–3.0 mg of AHLG with 10^9 cells from CML-BC at 20 C for 45 min The suspension was then centrifuged at 400 *g* for 20 min and the clear supernate incubated a second time with 10^9 cells from AML. The recovered AHLG was then centrifuged at 80 000 for 2 h

Lymphocyte absorption B lymphocytes from CLL were used as immunoabsorbent for anti TD AHLG the AHLG/cell ratio being 2 3 mg/2 $\times$ 10^9 lymphocytes Absorption procedures were performed as described above and the recovered AHLG was then centrifuged at 80 000 *g* for 2 h

Absorption elution Anti CLL AHLG (1 ml) was incubated for 1 h at 20 °C with B lymphocytes from CLL (2 8 mg AHLG 2 $\times$ 10^9 cells) previously washed in phosphate buffered saline (PBS) 3 times and either fixed in 0 1% formaldehyde for 5 min at 20 °C or in fresh suspension [39] The cells were washed 5 times in PBS in order to remove unbound protein and antibody elution was performed by resuspending the cells in 0.5 ml of sodium citrate buffer 0 12 M pH 3 for 5 min at 20 °C After centrifugation and recovery of eluted AHLG the cells were again resuspended in 0.5 ml of citrate buffer to produce a second batch of eluates, and the two pooled eluted AHLG were finally clarified by centrifugation at 80 000 *g* for 2 h

Protein determinations and cellulose acetate electrophoresis were performed on commercial and refined AHLG before and after myeloid or lymphoid absorption and after absorption-elution

AHLG reactions Fixed cell studies were made utilizing 20 °C methanol fixed smears appropriately washed in PBS incubated with AHLG at 20 °C for 1 h washed again in PBS and exposed to the immunofluorescent antiserum However most observations were made with fresh cell preparations incubated for 1 h with AHLG dilutions Admixtures of AHLG and cell suspensions were made with 50 µl of each reagent and left for 1 h at 4 C Three washes in fetal calf Hanks solution were performed subsequently and the final volume was adjusted to the initial 50 µl

These preparations were then incubated for an additional hour with 50 μl of a 1:5 dilution of rabbit anti horse immunoglobulins conjugated with FITC, washed 3 times and mounted between sealed coverslip. Finally, 6 cases of ALL, 6 of CLL and 6 of AML were also treated with FITC conjugated AHLG samples in direct, fresh suspension observation.

Controls were made by incubating lymphoid and myeloid cell suspensions with horse immunoglobulins and subsequently with anti horse FITC conjugated antibodies or by reacting lymphoid and myeloid cells with anti horse FITC-conjugated antibodies alone. In both cases no lymphoid fluorescence but only a weak particulate intracytoplasmic fluorescence of mature granulocytes could be observed.

Microscopic examinations were made with a Zeiss Photomikroscop II instrument housing both tungsten and mercury (HBO 200 W/4) illuminators. Transmitted excitation was performed with a short wave pass interference filter (KP 500) having a sharp cut off at 490–500 nm particularly suited for FITC. Barrier filters were the customary 500-nm types removing excitation irradiation. A dark ground condenser and iris equipped objectives were utilized. Epi illumination was performed with incident excitation furnished through an interference dividing plate (dichroic mirror) fitted with the same interference filter. Detailed morphological observations were made by examining the same cells by phase and fluorescence subsequently. Photomicrographs were made with Ektachrome high speed artificial light (tungsten 3200 K) films.

Results

When fixed lymphoblast preparations were inspected, whether with transmitted or incident excitation, a bright fluorescence involving the whole cytoplasm could be seen (fig. 1). However, a background staining was always present and sometimes troublesome. On the other hand, fresh suspensions, examined under epi-illumination, consistently showed a brilliant membrane fluorescence, sharply contrasting with the uniformly dark background (fig. 2). In addition, titres, taken as the highest reciprocal positive dilutions, were greater by magnitudes of 2 and 3 logs than with the fixed preparations, so that the fresh suspension procedure was adopted predominantly throughout these studies, and will be referred to when not stated otherwise.

Morphologically at low dilutions (1:50–1:5,000 with non absorbed AHLG), fluorescence was seen as involving the whole cell surface (fig. 3) or as a bright continuous equatorial ring surrounding the cell (fig. 4, 5). At these dilutions, frequent agglutination phenomena could be observed (fig. 6, 7), and erythrocyte agglutination up to titres of 1:160 could also be found. With greater dilutions, the fluorescent rings grew gradually less

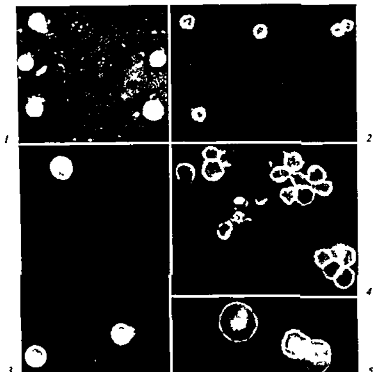


Fig 1 Fixed and dried preparation of lymphoblasts from ALL reacted with equine commercial AHLG and rabbit FITC-conjugated anti horse Igs, examined under transmitted excitation × 200

Fig 2 Fresh suspension of lymphocytes from CLL treated as in figure 1 and ex amined under incident illumination Dilution 1 5 × 10³ × 200

Fig 3 Fresh suspension of lymphoblasts from ALL treated with anti KL AHLG Dilution 1 5 × 10 × 200

Fig 4 Fresh suspension of lymphoblasts treated as in figure 3 Dilution 1 5 × 10³ Slight degree of agglutination × 200

Fig 5 Fresh suspension of lymphoblasts treated with anti TD myeloid absorbed AHLG Dilution 1 5 × 10² Typical continuous rim of fluorescence × 500

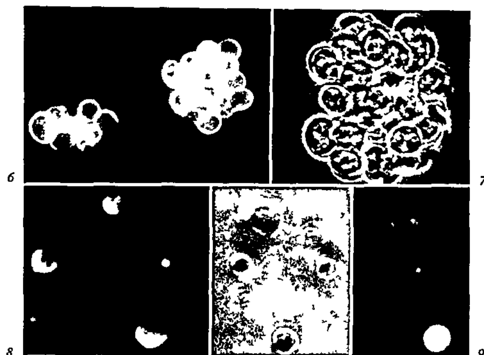


Fig 6 Fresh suspension of lymphoblasts treated with unabsorbed AHLG Dilution 1 5 $\times 10^2$ Strong agglutination $\times$ 200

Fig 7 Same preparation viewed in phase contrast

Fig 8 Fresh suspension of myeloblasts from AML treated with unabsorbed commercial AHLG Typical capping Dilution 1 5 $\times 10^2$ $\times$ 200

Fig 9 Fresh suspension of cells from AML reacted with anti CLL *myeloid absorbed* AHLG in the phase contrast (left) a promyelocyte can be seen in the upper part and a lymphocyte in the lower part After excitation with incident BV light (right) one can readily detect the lymphocyte surrounded by a continuous fluorescent ring while the myeloid cell is completely unstained $\times$ 300

bright, breaking into crescents and spots before total negativity Polar cap formation at high dilutions was an apparently active phenomenon, since it was clearly enhanced with time at 37 °C It affected more myeloid than lymphoid cells (fig 8), with a rough estimated ratio of 10 1 When these capped cells were examined under tungsten illumination and by phase, the capped region always corresponded to the region of the cell containing the Golgi complex and opposite to the nucleus, which, in these cells, had generally assumed an eccentric, polar location

Commercial AHLG With unabsorbed commercial AHLG 5 log (10^5) titres were obtained both with normal and leukaemic (from CLL) lym

phocytes, as well as with lymphoblasts from ALL However, a strong IMF of leukaemic (from AML and CML BC) and non leukaemic myeloid cells was also found, at titres not always differing significantly from lymphoid cells Because of these marked cross reactions, a first group of cases was reacted with commercial AHLG preabsorbed with myeloid cell suspensions, as described previously Greater specificity for lymphoid cells was obtained, but not exceeding 1 log in dilution titres as compared with myeloid cells The results of this first group of experiments is shown on table I

Refined AHLG The whole series of Behring's refined AHLG was then tested, and unabsorbed anti KL (Fab)., anti-TD and anti CLL showed best specificity in comparison to the other antisera (table II) and the same 1-log difference as the commercial myeloid-absorbed AHLG

Table I Immunofluorescence titres with commercial, myeloid absorbed AHLG

Type of leukaemia	Number of cases	Titres (rec of dilution)
CLL	10	2×10^5
ALL	8	2×10^5
Sézary s syndrome	1	2×10^5
AML	10	2×10^4
CML–BC	5	2×10^4

Table II Immunofluorescence titres with refined unabsorbed AHLG

Lymphocyte source	Titres (rec of dilution)		
	ALL	CLL	AML
Thoracic duct	5×10^4	5×10^4	5×10^3
Thoracic duct $(Fab)_2$	5×10^4	5×10^4	5×10^3
Thoracic duct (membranes)	2×10^4	2×10^4	5×10^3
CLL	5×10^4	5×10^4	5×10^3
Cultured lymphoblasts	5×10^4	5×10^4	5×10^3
Cultured lymphoblasts $(Fab)_2$	5×10^4	5×10^4	5×10^3
Thymus	5×10^4	5×10^4	5×10^4
Spleen	5×10^4	5×10^4	5×10^4
Lymph node	5×10^4	5×10^4	5×10^4
Tonsil	5×10^4	5×10^4	5×10^4

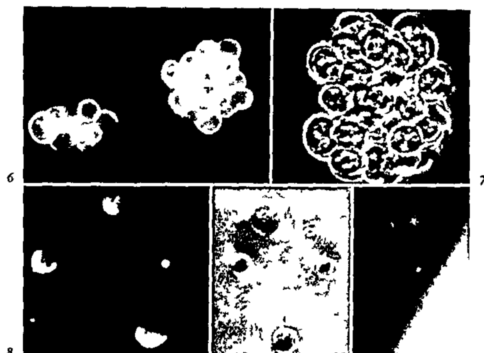


Fig 6 Fresh suspension of lymphoblasts treated with unabsorb tion 1 5 × 10² Strong agglutination × 200

Fig 7 Same preparation viewed in phase contrast

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Table IV Effect of different absorptions on antibody content and lympho-myeloid discrimination of three AHLG preparations

Type of AHLG	Proteins mg/ml	Antibody recovered, %	Lympho-myeloid discrimination
Anti-TD			
Unabsorbed	2.5		fair
B lymphocyte-absorbed	1.5	60	fair
Myeloid-absorbed	1.81	72	excellent
Anti CLL			
Unabsorbed	2.8		fair
B lymphocyte-absorbed/eluted	0.31	11	fair
Myeloid absorbed	1.30	46	excellent
Anti-KL (Fab)₂			
Unabsorbed	2.5		fair
Myeloid absorbed	1.85	74	excellent

Cellulose acetate electrophoresis performed on the refined AHLGs were compatible with pure Ig preparations, with varying amounts of γ_1- and γ_2-fractions, the latter comprising 98% of total Ig in anti-TD AHLG. Protein determinations performed before and after absorption and after absorption elution showed that approximately 60% of AHLG could be recovered after myeloid absorption, ranging from 46 to 74% according to the type of AHLG used. The percentage of recovered antibody after lymphocyte absorption was 60%, but only 11% after absorption-elution (table IV).

Discussion

Our experiments demonstrate that AHLG will react, in direct and indirect IMF tests, not only with mature lymphocytes, but also with the immature lymphoblasts of ALL. Thus, under this aspect, AHLG behaves differently from the monospecific antigranulocyte sera of MAHMOUD *et al* [22], which appear to possess more specific cytotropic properties. In fact, cultured lymphoblastoid cells and/or their membranes are one of the best antigenic sources for raising ALG [2, 28].

Table III Immunofluorescence titres with anti-KL $(Fab)_2$, anti-TD and anti-CLL, myeloid-absorbed AHLG

Type of leukaemia	Number of cases	Titres (rec. of dilution)
CLL	10	5×10^4
ALL	10	5×10^4
AML	10	5×10

When the three mentioned antisera were preabsorbed with myeloid cells, there was a much more marked gradient in lymphoid-myeloid cell fluorescence (3 logs), with practically no loss in lymphoid-positive titres (table III, fig. 9). Direct IMF tests performed with FITC-conjugated anti-CLL, anti-TD, anti-KL and anti-MX AHLG diluted 1:4 did not show relevant differences in fluorescent patterns, myeloid and lymphoid cells appearing both as bright continuous rings; a slight gradient could however be observed among lymphoid and myeloid cells, in spite of the very low dilution titre.

When B lymphocytes absorbed anti-TD AHLG was reacted with peripheral blood lymphocytes from normal donors there was a close correlation between the percentage of AHLG-stained cells and the E-rosetting lymphocytes. However, when the same B-lymphocyte-absorbed AHLG was incubated with leukaemic myeloid and lymphoid cells, no amelioration of the lymphoid-myeloid gradient, in comparison to unabsorbed anti-TD AHLG, could be observed. Furthermore, all our cases of ALL reacted similarly with anti-TD B-lymphocyte-absorbed, anti-CLL and anti-KL AHLG.

Absorption-elution. The recovery of AHLG from lymphocyte-absorbed/eluted anti-CLL AHLG was approximately 10% in comparison to the initial preparation. when the eluted anti-CLL AHLG was tested on lymphoid and myeloid cells, no significant differences in fluorescent patterns, percentage of positive cells or intensity of fluorescent staining could be found, both cell preparations being still positive at $1:5 \times 10^3$ dilutions There was instead a loss in lymphoid-positive titres in comparison to the unabsorbed anti-CLL AHLG (from $1:5 \times 10^4$ with the latter to $1:5 \times 10^3$ after absorption-elution) No correlation with Ig-bearing peripheral blood lymphocytes and eluted anti-CLL AHLG-stained lymphocytes could be obtained

Table IV Effect of different absorptions on antibody content and lympho-myeloid discrimination of three AHLG preparations

Type of AHLG	Proteins mg/ml	Antibody recovered, %	Lympho-myeloid discrimination
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Unabsorbed	2.5		fair
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Anti CLL			
Unabsorbed	2.8		fair
B lymphocyte absorbed eluted	0.31	11	fair
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Discussion

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IMF reactions were obtained with fixed and dried preparations, but were much brighter and with higher titres when membrane fluorescence was sought for in the living state. It would seem accordingly that similar, if not identical, 'lymphoid' antigens are recognized by AHLG in the cytoplasm and on the surface of these cells. Although variations of ALG binding in lymphoid cell cultures have been detected and interpreted as indicators of changing membrane antigen expression [31], similar phenomena were extremely rare in our experiments.

ALL has been shown to be highly heterogeneous with regard to the T or B nature of the leukaemic lymphoblasts [6, 11], often lacking reactivity even with a wide battery of immunologic markers [6]. Its immunologic classification is still considered uncertain [26], and it has been even hypothesized that it may not derive from the lymphoid developmental pathway [6]. Although the main object of our research was not to investigate the increasingly moot point of their T or B nature, but rather to identify a common lymphoid antigen, it should be remarked here that no difference in IMF reactions of ALL cells were found when using unabsorbed ('T + B') or anti-TD B-absorbed ('anti-T') AHLG and its subvarieties.

The vigorous cross-reactions with immature myeloid cells, including basophilic, agranular myeloblasts, obtained with unabsorbed AHLG, were not unsuspected because of the heterologous origin of the AHLG. Heteroantisera, because of the large number of antigenic differences involved in immunizing across species, must be extensively absorbed to make them specific for a particular differentiation antigen [14], such as the 'lymphoid' antigen(s). AHLG has been accordingly shown to interfere with haemopoietic stem cells [10] and the relevant antigens have been found in epidermal cells, L cells and mouse fibroblasts. More recently, common antigens have been demonstrated between mouse brain cells, haemopoietic stem cells (CFU) and lymphoma cells [6, 13]. Finally, a common antigen has been postulated by TAYLOR [33] in order to explain the lymphoid-myeloid cross reactivity he found reacting ALS in tissue sections. A common, multipotential stem cell is generally postulated at the origin of the so-called lymphomyeloid complex. These immunobiologic properties will perhaps give rise to great difficulties when using the test to assess the nature of so called undifferentiated leukaemias.

Of the two different approaches undertaken to eliminate myeloid cross-reactivity, namely absorption-elution of the *relevant antibodies* [18] and exhaustion by preabsorption of the *cross-reacting antibodies*, no sig-

nificant advantage could be obtained with the former procedure, notwithstanding the considerable (19 times) enhancement of immunosuppressive potency reported by YAMANA *et al* [39] On the contrary, preabsorption with immature and/or mature cells from AML and CML-BC was capable of preventing cross-reactions, with a difference of 3 logs, when the absorbed AHLG belonged to the anti-TD, anti CLL and anti KL (Fab). subvarieties This we feel, is the fundamental approach to bring the test amoung routine haematologic investigations

Although collateral to the main object of these investigations, the phenomenon of polar capping with AHLG of the lymphoid and especially of the myeloid cells was so evident to warrant a short commentary Cap formation was originally described in mouse lymphocytes when they were reacted with fluorochrome-conjugated anti immunoglobulin antisera [34], and regarded as a two-step process, involving the aggregation of the ligand receptor complexes into patches, and the subsequent concentration of the same in a polar crescent or pear like formation opposite the nucleus and prospicient to the Golgi area [8, 37] as shown also electron microscopically [7] This last phenomenon appears to be metabolically driven, since dead cells do not cap [8] nor do living cells in the cold [8, 21] or after exposure to drugs blocking glycolysis oxidative phosphorylation and microtubular activity [8 38] It has been speculated that capping may derive from cell movement the cell crawling out of the lattice of complexes 'somewhat like a snail emerging from its shell [19] or, alternately, from superficial movement of the membranes and/or from membrane flow independent from actual translation This latter view has been confirmed recently [38] In any case, although originally described in immunoglobulin surface bearing cells it is certainly a more general phenomenon and has already been described in non lymphoid cells such as mouse fibroblasts [8] and guinea pig [9] and human [3] basophils exposed to anti IgE antibodies Immature leukaemic myeloid cells having reacted with ALG have been shown to display the same phenomenon with greater intensity than the lymphoblasts probably because of their greater intrinsic motility

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A Raised Incidence of HL-A2 plus HL-A9 and other Anomalies of the HL-A Antigens of Patients with Leukaemia

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Key Words HL A Leukaemia

Abstract In 54 patients with leukaemia a raised incidence of HL-A9 was noted as well as a markedly increased association between this antigen and HL-A2 This occurred most frequently in patierts with chronic myeloid leukaemia As HL A2 and HL-A9 are both antigens of the first series it has been suggested that the predis position to develop leukaemia is controlled by a recessive gene closely linked to the first HL-A locus and in a linkage disequilibrium with HL A2 and HL-A9 5 patients also showed definite changes between antigens of the same series, whilst others suffered a partial or total loss in antigenicity Lymphocytes from 145 controls did not behave in this way, though other patients receiving radiotherapy also 'lost' antigens So it was postulated that such changes resulted from the treatment of the disease rather than the disease itself

There have been reports from various workers concerning the correlation between HL-A antigens and the occurrence of certain diseases or the susceptibility of individuals to develop different diseases [1, 2] The most conclusive results were those showing a correlation between HL-A27 and ankylosing spondylitis [3] and HL-A5, W5 [1, 4–7] with Hodgkin's disease and other types of lymphoma

Similar studies have been carried out on the HL-A types of patients with leukaemia with conflicting results The largest study carried out was that of the Histocompatibility Testing Workshop in 1972 which concluded that there was no relationship between HL-A and any form of leukaemia However, individual workers [2, 6, 8, 9] have suggested an association between HL-A2 and the disease, whilst WALFORD [10] has shown a positive association between HL-A2, HL-A12 and childhood acute leukaemia, but a negative association between HL-A1 and the same disease

PEGRUM *et al.* [11] found a positive association between HL-A1, HL-A3 and acute lymphoblastic leukaemia.

It may be, however, that far from the HL-A genotype affecting the likelihood of an individual developing leukaemia, the reverse is true, and the development of the disease alters the antigenicity of the cells. It has already been shown [12–14] that the ABH antigens are weakened on the leucocytes of individuals with leukaemia. A further possibility is that, though the disease itself may have no effect upon the antigenicity of the cells, the treatment these patients are receiving may result in alterations to the antigens, or to a partial or total loss in antigenicity. It is known that both radiotherapy and certain drugs including at least one, cytosine arabinoside, used in the treatment of leukaemia can cause chromosome aberrations [15] which may result in antigen changes.

During a recent investigation in this laboratory of patients with leukaemia for the presence of lymphocytotoxins in the serum [16], the patients were also typed for HL-A antigens with these theories in mind. An attempt was made to determine whether there were any associations between HL-A antigens and leukaemia in this population, and to see whether any such associations corroborated one of the earlier reports. At the same time changes in antigenicity were looked for, and an attempt was made to determine the cause of these changes.

Methods

Lymphocytes were obtained from defibrinated blood by centrifuging through a high density solution of Ficoll and Triosil as described by HARRIS and UKAEJIOFO [17]. They were typed for HL-A antigens by the two stage microlymphocytotoxicity test of TERASAKI and MCCLELLAND [18]. The antisera used were obtained from the National Institutes of Health at Bethesda, Md., and covered antigens HL-A1, 2, 3, 5, 7, 8, 9, 10, 11, 12 and 13. A panel of 30 sera were available of which 20 were thought to be monospecific.

Lymphocytes from 54 patients with leukaemia were typed on each visit to the hospital, 41 of them on several occasions and at different stages of their disease. The patients were divided into the groups acute, sub-acute, and chronic myeloid leukaemia, and acute and chronic lymphatic leukaemia (table I). As controls, lymphocytes obtained from all the members of the laboratory were typed repeatedly with consistent results, and this was considered to be a normal population albeit a small one. Patients with other diseases were also typed repeatedly on visits to the out patient clinics, and thus 145 individuals made up the control populations. The results were analysed using the χ^2-test with Yates correction [19] to test for the significance of the antigen frequency.

Table I Patients typed for HL-A antigens

Type of leukaemia	Number tested
Acute myeloid	13
Sub-acute myeloid	2
Chronic myeloid	9
Acute lymphatic	6
Chronic lymphatic	18
Acute, undifferentiated	1

Table II Results of HL-A typing

Antigen	Occurrence in leukaemia patients (n = 54), %	Occurrence in control group (n = 145), %	p value
HL-A1	45	46	>0.5
HL-A2	61	54	>0.1
HL-A3	13	26	>0.01
HL-A5	50	35	<0.01
HL-A7	26	31	<0.5
HL-A8	47	44	>0.5
HL-A9	42	16	<0.001
HL-A10	0	3	>0.5
HL-A11	0	8	>0.5
HL-A12	23	24	<0.5
HL-A13	16	5	<0.05

Results

There were problems encountered in the typing of these patients. It has already been reported [20] that patients suffering from leukaemia are extremely difficult to type, since the lymphocytes become very fragile resulting in a high background count, which at times, can mask the true HL-A types. Added to this is the fact that these patients receive frequent blood transfusions and may have transfused lymphocytes circulating in their blood. This may lead to anomalous results, such as three antigens of the same series which is genetically impossible [21] in normal individuals, although it might occur in a chromosome duplication. Furthermore, most

Table III Frequency of individuals with HL A2+HL A9

Population	Total	%	Significance of being different to control
Control	7/145	5	
Total leukaemia	15/54	28	p<0 001
Acute myeloid	*3/13*	*23*	*p<0 01*
Sub-acute myeloid	1/2		
Acute, unidentified	1/1		
Chronic myeloid	6/9	67	p<0 001
Acute lymphatic	1/6	17	p<0 05
Chronic lymphatic	3/18	17	p<0 05

of these patients were receiving repeated doses of cytotoxic drugs and in some cases radiotherapy, both of which destroy cells and may give a high background count, or alternatively may destroy the antigenicity of the cells However, despite these difficulties, when an analysis of the results was made, three facts emerged (1) a significant difference in the incidence of three HL-A antigens (table II), (2) a high incidence of HL-A2 occurring with HL-A9, especially amongst those patients with chronic myeloid leukaemia (table III), and (3) evidence for antigen changes in 5 patients with leukaemia (table IV)

As table II illustrates HL-A3 was found in 13% of the leukaemia patients compared with 26% of the controls (p = 0 01–0 02), whilst HL-A5 was found in 50% of the leukaemic population compared with 35% of the controls (p<0 01) However, the antigen HL-A9 had the most markedly altered frequency in the leukaemia population, occurring in 42% of these patients but only 16% of the controls (p<0 001)

Further analysis was carried out in order to detect any combinations of antigens that were particularly frequent in the leukaemia population. Certain combinations of antigens, one from each series, e g HL-A2 with HL-A12, are common in the general population and were found as expected in this survey In addition, HL-A2 was found occurring with HL-A9 in 15 out of the 54 patients (28%) compared with 7 of the 145 controls (5%) (p<0 001) The frequency of this combination was significantly raised in all forms of leukaemia, but most especially in the chronic myeloid leukaemias where 6 out 9 patients typed were HL-A2 plus

Table IV Changes in HL-A typings

	First series		Second series		
Patient 1					
Original type	HL-A2,	HL-A9	HL-A12,	X_2	
				↓	
Intermediate type	HL-A2,	HL-A9	HL-A12,	*HL-A5*,	*HL-A8*
				↓	
Final type	HL-A2,	HL-A9	HL-A12,	HL-A5	
Patient 2					
Original type	HL-A1,	*HL-A2*	HL-A5,	*HL-A7*	
		↓		↓	
Intermediate type	HL-A1,	*HL-A3*	HL-A5,	*HL-A8*	
				↓	
Final type	HL-A1,	*HL-A3*	HL-A5,	X_2	
Patient 3					
Original type	HL-A1,	*HL-A9*	*HL-A5*,	HL-A8	
		↓	↓		
Final type	HL-A1,	*HL-A2*	*HL-A7*,	HL-A8	
Patient 4					
Original type	*HL-A2*,	HL-A9	HL-A5,	*HL-A8*	
	↓			↓	
Intermediate type	*HL-A2*,	HL-A9	HL-A5,	*HL-A7*	
	↓			↓	
Final type	X_1,	HL-A9	HL-A5,	X_2	
Patient 5					
Original type	HL-A1,	*HL-A2*	*HL-A5*,	HL-A7	
		↓	↓		
Final type	HL-A1,	*HL-A3*	*HL-A8*,	HL-A7	

HL-A9 ($p<0.001$) *Those individuals of the control population,* 7 in all, in whom these two antigens occurred together, were examined more closely and were all found to be suffering from some form of malignant disease, either carcinoma of the bronchus, lymphosarcoma or reticulum cell sarcoma

Table IV shows the 5 patients in whom there is definite evidence for antigen changes In each case the change is between antigens of the same series, and where there is more than one change taking place the pairs of

Table V Results of HL-A typing on patient with ALL

No of typing	First series						Second series				
	HL-A1	2	3	9	10	11	HL-A5	7	8	12	13
1	–	–	–	–	–	–	–	–	–	–	–
2	+	–	+	–	–	–	–	–	+	–	–
3	–	–	–	–	–	–	–	–	+	–	–
4	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–

antigens involved belong to different series, suggesting that in each case one chromosome of the pair is affected Besides these patients with definite antigen changes it was found that several of the patients suffered a loss, sometimes total, of antigenicity This was particularly noticeable amongst patients receiving radiotherapy treatment One boy with acute lymphoblastic leukaemia on an intensive regime of radiotherapy illustrates this well (table V) Several of the patients studied, with other diseases, were attending the radiotherapy out patient clinic, and these patients were similarly difficult to type and subject to antigen loss

Discussion

The raised frequency of HL-A9 in the leukaemia patients studied here has not previously been reported Indeed BATCHELOR *et al* [22] found no individuals amongst their acute lymphoblastic leukaemia patients who were HL-A9 positive, whereas two patients with this type of leukaemia have typed repeatedly for HL-A9 in this study However, this is not significantly high, and it is the incidence of HL-A9 in the myeloid leukaemias that raises the incidence so greatly in this population In the past no associations have been found between myeloid leukaemias and HL-A antigens [2, 9], and it must be pointed out that this population is small However, it is interesting to note that the incidence of HL-A9 is also raised in the Jewish population (51%) [23], a group in which the incidence of leukaemia is elevated [24, 25], a fact which supports the suggestion that HL-A9 may be associated with the disease

A more interesting finding is the raised frequency of HL-A2 in association with HL-A9, in these patients, especially those with chronic mye-

loid leukaemia Unlike the previously noted associations of antigens, HL-A2 and HL-A9 are antigens of the same (first) series of the HL A system They must therefore be inherited one from each parent and would be expected to occur together rarely by chance, since HL-A9 is not a common antigen especially in the Caucasian population (16%) In the Jewish population the frequency of HL-A2 (33 5%) is lower than among Caucasians (54%) Nevertheless this antigen occurs frequently enough to be found in association with HL-A9 more often than in other races The high incidence of these two antigens occurring together in leukaemia, especially amongst the chronic myeloids, suggests that the presence of both in an individual predisposes him to develop leukaemia especially of the myeloid type This type of inheritance would be in accordance with the fact that, whereas the incidence of leukaemia amonst sibs is higher than might be expected from its occurrence in the general population, it is rarely found in different generations of any one family

It has been suggested [26] that it is not the HL-A antigens themselves that predispose an individual to develop leukaemia, but another gene, possibly connected with the efficiency of the immune response and closely linked to HL-A, that is responsible If crossing-over were infrequent between this gene and HL A then it would be expected to occur more frequently associated with one or more HL-A antigens, in the same way that a linkage disequilibrium exists between certain HL-A antigens of the first and second loci If one were to accept this hypothesis it could be further suggested that this gene may be found more closely linked to the first HL-A locus, in a linkage disequilibrium with HL-A2 and HL-A9 If it were a recessive gene then the predisposition to develop leukaemia would only be present in homozygotes, and when HL-A2 and HL-A9 were found together in an individual, the likelihood of that individual developing the disease would thus be increased

It seems unlikely that the antigen changes have any connection with the predisposition to develop the disease, since it is not a case of the antigens being changed to those favourable to the disease What seems more probable is that the treatment received by these patients is responsible for the changes especially as it is already well known that radiotherapy and certain drugs including cytosine arabinoside used in leukaemia therapy, can cause chromosome aberrations [15] The most likely alteration is the deletion of all or part of a chromosome, which would certainly explain why antigens and sometimes total antigenicity, are lost in many of the patients who have received radiotherapy Certain chromosomes are more

susceptible to such effects than others Thus, it is reasonable that the chromosomes controlling the HL-A antigens might be so frequently involved It may be that certain drugs can cause mutations to the genes controlling the antigens of the HL-A system, and that these mutations result in alterations to the amino acid sequence and thus to differences in the antigens expressed The final effect may be the deletion of the portion of the chromosome containing one or both sub-loci of the HL-A system and thus antigen loss

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Alterations in Erythropoiesis Preceding Leukemia[1]

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Key Words Aregenerative anemia · Erythropoiesis · Preleukemia

Abstract An attempt was made to study preleukemic changes in bone marrow cell proliferation Seven patients with hypercellular marrows and aregenerative anemia were studied Five of them could be followed to autopsy, several years after the kinetic studies, all died with a picture of leukemia Total bone marrow cell numbers, erythroblast generation times, and erythrocyte production were estimated with ^{59}Fe Despite hypercellularity, the total erythroblast number was not significantly increased Erythrocyte production and life span were both decreased, and erythroblast generation times were significantly longer than normal

Previous studies of patients with aregenerative anemia, or so-called aplastic anemia [1, 9, 12, 14], suggest that some of these cases are of a preleukemic nature However, there are no unequivocal preleukemic stigmata, the literature has been reviewed by REIZENSTEIN and LAGERLÖF [9] There are few cytokinetic studies in man of the changes in the cellular growth patterns preceding malignancy There are indications that patients with an aregenerative anemia of a sideroblastic type with a hyperplastic bone marrow will eventually develop leukemia, in some instances not until after many years [3, 4, 9] Certain alterations may be present not only in the myeloid cells, which may become malignant later on, but also in other bone marrow cells, which will usually not show any cytologic signs of malignancy This would explain why anemia and thrombocytopenia often precede leukemia [9] The present purpose was to study alterations in the erythroblast growth rate in patients with aregenerative anemia, some of which can be assumed to

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develop leukemia later In an independent study, such patients are compared to those with aregenerative anemia less probable to develop leukemia later [12] In none of these patients were any signs of leukemia noted at the time of the study

Material and Methods

Seven patients with aregenerative anemia, hyperplastic bone marrow, and more than 14% sideroblasts in the bone marrow were studied They are described in tables I and II Three patients (cases No 2, 3, 5) had small numbers of myeloblasts in the peripheral blood, but no patient had a bone marrow with a dominating population of neoplastic myeloblasts In no patient was the initial bone marrow suspected of being acute leukemia

The highest percentage of sideroblasts as defined below which was found in any control bone marrow, was 14% Erythroblasts were designed as sideroblasts when they contained more than 6 granules, with or without ring form arrangement Bone marrow sections, sideroblast stains, radioiron uptake studies, blood volume determinations, and whole body bone marrow cellularities were determined as previously described [9–11].

Whole body erythrocyte production per day was also calculated, but in the previous studies it was based on an assumed steady state and thus on the red cell survival time [13] In the present patients this assumption was not justified Instead, the erythrocyte production was calculated on the basis of the following assumptions [12] (1) An approximately linear relationship between the erythrocyte production and erythrocyte radioiron incorporation may be assumed. This assumption is made only for the limited range of *radioiron incorporation values seen in patients with aregenerative anemia* (2) Normal erythrocyte survival is 120 days and normal ^{59}Fe erythrocyte incorporation is 80% of the given dose (50 to 97 000 cps)

Table I Clinical description of the patients at time of study

Case No	Sex	Age years	Hb, g/100 ml	Platelets, $\times 10^3/\mu l$	Serum iron, $\mu g/ml$	Total iron binding capacity $\mu g/ml$	Radioiron incorporation into erythrocytes, % of given dose
1	M	60	11	3	NF	NF	4.2
2	M	59	11 8	110	0 205	0 269	17 5
3	M	68	10 9	35	0 115	0 156	0 6
4	M	64	9 1	316	0 023	0.202	42 0
5	F	42	10 3	166	0 159	0 299	11 3
6	M	71	10 9	269	0.253	0 348	0 8
7	M	62	8 8	130	0 215	0.284	34 6

NF = Value not found

Table II Clinical description of the patients at time of study (continued)

Case No	Differential count, %									Total body hemoglobin, g[1]		Blood volume, l[1]		Autopsy diagnosis
	WBC, ×10³/µl	meta	segm	eos	baso	lymph	mono	plasma	myelobl.	found	expected	found	expected	
1	0.5	NF	NF	NF	NF	NF	NF	NF	NF	431	680	NF	NF	myeloblastic leukemia
2	3.2	1	2	0	1	38	47	4	7	423	659	5.2	5.0	myeloblastic leukemia
3	42.8	13.5	29	1.5	14.5	18.5	13.5	0	9.5	238	544	2.9	4.1	myeloblastic leukemia
4	8.2	4	68	0	0	19	9	0	0	588	774	7.2	5.8	myeloblastic leukemia
5	2.1	4	23	2.5	0.5	29.5	36	0.5	4	393	628	4.4	5.3	alive
6	10.4	18.5	52.5	4	0	25	0	0	0	357	852	4.7	5.8	no autopsy
7	3.2	1.5	50	1	0.5	35	12	0	0	384	690	5.2	5.2	incip. myelobl. leukemia

NF = Values not found

[1] Determined with alveolar CO method [10]

Under these assumptions, the number of erythrocytes produced per kilogram body weight was calculated to be

$$\frac{\text{incorporation}_{\text{normal}} \times \text{erythrocyte production per kg}_{\text{normal}}}{\text{incorporation}_{\text{found}}}$$

In the previous studies [11, 12], the whole body bone marrow cellularity was calculated by measuring the mean bone marrow cell radioactivity, and by assuming that the total bone marrow radioactivity equals the total radioactivity incorporated into erythrocytes [11]. In the present patients, this assumption was not justified either. It is known [5–8] that in aregenerative and secondary anemia iron is incorporated into bone marrow reticuloendothelial cells and retained there without being incorporated into erythrocytes. For this reason no individual estimation could be made of the whole-body bone marrow radioactivity, which could only be assumed to average 66% of the total injected radioactivity [2].

Based on the estimated whole-body bone marrow cellularity and whole-body erythrocyte production, as well as on the bone marrow erythroblast differential count, erythroblast generation times could be calculated according to the formulae described previously [13], based on the additional assumptions that there is one heteroplastic mitosis in each of four maturation stages between proerythroblast and reticulocyte, and that intramarrow cell death and maturation without division even out. Where the erythrocyte production (E_{in}) is known,

$$E_{in} = \frac{2\,N_p}{t_p},$$

where N_p is the number of polychromatic erythroblasts, and t_p their generation time. In a similar manner, generation times of preceding maturation stages can be calculated [13].

Results

Clinical. Six of the 7 patients were men older than 58 years (table I). Their anemia was moderate, only 3 of the patients had less than 10 g Hb/100 ml, but the total body hemoglobin was reduced to between 42 and 67% of the calculated normal. One of the patients had granulocytopenia, and 3 had moderate thrombocytopenia. Differential cell counts were generally normal, free of leukemic blast cells or immature cells, and 27–71% of the circulating white blood cells were stab cells or segmented neutrophils. All of the 7 patients studied could be followed up 1–7 years after the study. At this time, 1 is alive with anemia, and 5 have died with an autopsy diagnosis of leukemia. In one case, no autopsy could be performed.

Erythroblast numbers. By definition, all patients included in the present study had a hypercellular bone marrow with a reduced number of fat cells. The cell population was generally dominated by the myeloid cell series and

Table III. Erythroblast percentage and number and erythrocyte production (means ± SE)

	Number of patients	Total nucleated marrow cells, %	Number of erythroblasts, $\times 10^9$/kg	Production, $\times 10^7$ cells/kg/h	Erythrocyte mass, $\times 10^9$ cells/kg
Aregenerative anemia	7	8.9 ± 4.3	2.0 ± 1.03	2.4 ± 0.7	11.2 ± 2.54
Comparison patients	12	17 ± 1.04	3.4 ± 0.026	9.1 ± 1.7	26.4 ± 4.7

Table IV. Generation times in hours (mean ± SE)

	Number of patients	Proerythroblasts	Basophilic erythroblasts	Polychromatic erythroblasts
Aregenerative anemia	7	27.0 ± 10.6	56.8 ± 24.3[1]	79.0 ± 24.9[2]
Comparison patients [11, 13]	12	10.9 ± 0.6	16.0 ± 1.0	26.0 ± 1.6

[1] $0.05 > p > 0.01$.
[2] $0.01 > p > 0.001$

the erythroblast percentage in the bone marrow differential count was reduced in all patients except one (Case No 7). The erythroblast percentage averaged 17 in control patients [11, 13] and it averaged 8.9 in the present patients. *This difference was not significant statistically. Similarly, the whole* body erythroblast numbers were frequently, though not statistically reduced (mean values are shown in table III). Values for comparison patients have been given previously [11, 13], and are also included in tables III and IV.

Erythrocyte production. By definition, the radioiron incorporation into erythrocytes was reduced significantly ($0.01 > p > 0.001$) and substantially. In fact, only an average of 16% of the radioiron were incorporated. Accordingly, the total daily erythrocyte production was also significantly ($0.01 > p > 0.001$) reduced (table III). The mean red cell survival time (38 ± 3 days) was reduced ($0.01 > p > 0.001$) in the present patients, when the survival time was measured with the CO hemoglobin method. All patients,

except one, had calculated polychromatic erythroblast generation times far beyond the normal range The calculated mean erythroblast generation times for basophilic and polychromatic erythroblasts were thus significantly ($0.05 > p > 0.01$ and $0.01 > p > 0.001$) increased (table IV) The proerythroblast generation times were not significantly prolonged, but 4 out of 7 patients did have markedly prolonged generation times

Radioiron distribution in the bone marrow preparation Bone marrow cells seemed more fragile than in comparison patients [11] who had an average of 3 3 cps/ml of the washed bone marrow cell sample and 0 4 cps/ml supernate and washing fluid, respectively A larger portion (0 49 cps/ml) of the iron is found in the extracellular fluid in the present patients, in comparison to the portion (0 76 cps/ml) in the washed marrow cells In a previous study [9], 4 out of 8 patients with aregenerative anemia and *hypercellular* marrow sections had leukemia at autopsy, as compared to none of 6 patients with a *hypocellular* marrow The present study, where all patients with a hypercellular marrow, whose autopsy was obtained had developed leukemia, seem to confirm the previous suggestion made by REIZENSTEIN and LAGERLÖF [9] that aregenerative anemia may be of a preleukemic nature

Discussion

The basic observation in this paper is that a group of patients with a high probability to develop leukemia show a reduced incorporation of ^{59}Fe into circulating red cells without having a reduced number of bone marrow cells This observation can be interpreted in cell-kinetic terms It is not an easy task to measure erythroblast generation times *in vivo* in patients, where erythrocyte production and destruction are not in a steady state We recognize, therefore, that the present kinetic values are to be regarded as tentative because of the three assumptions required for the calculation of the generation times, and because of the relatively large amounts of extracellular bone marrow radioiron, possibly explained by larger amounts of radioiron being incorporated into fragile reticuloendothelial cells If this iron is derived from completely disrupted cells, it does not affect the generation times However, if it derives from partly broken cells, the nuclei of which are counted as cells, then the generation times given above may be too long Since the total numbers of bone marrow cells were increased and erythroblast percentages reduced, the whole body numbers of erythroblasts remained within normal limits in most patients (table III)

The present results suggest, but do not yet prove that a prolongation of generation times actually occurs in the form of aregenerative anemia, which, we believe, is of a preleukemic nature However, the present results are confirmed again in a separate study [12], where it is also indicated that generation times are not prolonged in the hypoplastic form of aregenerative anemia which develops into leukemia less frequently

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Correlation in Hemodialysis Patients and Renal Allograft Recipients between Percent T Lymphocytes in Peripheral Blood and *in vitro* Lymphocyte Responses to Nonspecific Mitogenic Agents[1]

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Key Words Cellular immunity Hemodialysis Lymphocyte stimulation Renal transplantation T Lymphocytes

Abstract Both hemodialysis and renal allograft recipients have a significantly reduced number of total T lymphocytes per microliter of blood Simultaneous in vitro lymphocyte responsiveness to phytohemagglutinin (PHA) and pokeweed mitogen (PWM) revealed in the normal subjects, a positive correlation ($r = +0.427$) between percent T lymphocytes and PHA and a negative correlation ($r = -0.525$) between percent T lymphocytes and PWM Such trends were not observed in the hemodialysis patients and transplant recipients Thus, the enumeration of lymphocytes as T cells appears to provide no clear indication of their functional capacity to respond to mitogenic stimulation in these two categories of patients

Depressed cellular immunity has been reported in uremic patients [2, 8, 9, 11, 14] and in renal allograft recipients [7] We have studied the number of T lymphocytes in these two groups of patients in an effort to explain depressed cellular immunity in the two categories of patients at least partly on the basis of reduced numbers of T lymphocytes Functionally, T lymphocytes primarily respond to phytohemagglutinin (PHA) while pokeweed mitogen (PWM) stimulates primarily B lymphocytes [12, 15] Thus, patients' lymphocytes were also studied simultaneously in *in vitro* cultures for their response to PHA and PWM to establish

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also the correlation between T lymphocyte number and these *in vitro* parameters of lymphocyte functional capacity

Materials and Methods

Patients A total of 19 uremic hemodialysis patients and 14 renal allograft recipients and 17 normal subjects were studied for T rosettes and *in vitro* response of lymphocytes to PHA and PWM Uremic patients were hemodialyzed by Kiil parallel flow dialyzer 2–3 times a week for a total of 24–30 h/week The duration of hemodialysis varied from 2 to 59 months with a median of 18 months Renal allograft recipients were on varying regimens of immunosuppressive therapy (Imuran and Prednisone) and had a functioning allograft 2 weeks to 46 months (median 4 months) after transplantation All patients had stable renal function with a serum creatinine of <2 mg%/₀ at the time of testing

Lymphocyte preparations Lymphocytes purified by the Ficoll Isopaque technique [1, 10] were utilized both for the study of T rosette formation and for the study of *in vitro* lymphocyte responses to PHA and PWM The number of monocytes in the purified preparations was similar (1–6%) for both normal subjects and patients The preparations obtained from patients did at times contain more polymorphonuclear leukocytes than those of normal subjects However, no preparation was utilized for either rosette formation or *in vitro* response to mitogens if polymorphonuclear cell contamination exceeded 10% of total lymphocyte count Total leukocyte counts and differentials were simultaneously made from an aliquot of blood to calculate total lymphocytes present per microliter of blood

T rosette assay T lymphocytes were determined by the rosette technique described by FARID *et al* [6] Briefly, 1×10^6 purified lymphocytes suspended in 0 25 ml of medium 199 were mixed with an equal volume of 1% suspension of SRBC in 12×75 mm plastic tubes Tubes were incubated for 15 min at 37 °C and then centrifuged at 200 g for 5 min at 4 °C Additional incubation was carried out for 1 h at 4 °C At the end of incubation most of the supernatant was removed and the pellet was resuspended by gentle shaking A rosette was defined as a lymphocyte with four or more adherent SRBC The percentage of rosette forming cells was determined by counting 200 lymphocytes per tube and is presented as the mean value of triplicate determinations per patient The total number of T lymphocytes per microliter of blood was determined by multiplying the percent T lymphocytes present and the total lymphocyte counts per microliter of blood

In vitro *response to mitogens* The *in vitro* reactivity of lymphocytes to PHA and PWM was studied using culture conditions similar to our previously described micromethod for the mixed leukocyte culture test [10 11] A total of 100 000 purified lymphocytes were cultured in 0 1 ml medium 199 Hepes in the presence of 0 05 ml heat inactivated normal AB plasma and 0 01 ml of either a 1 2 dilution of reconstituted PHA M (Difco Laboratories) or a 1/16 dilution of reconstituted PWM (Gibco Laboratories) These optimum concentrations for each mitogen were determined by a prior dose and time response study [SENGAR and HARRIS unpublished data] All cultures were labelled after 48 h of incubation with 0 8 μCi of

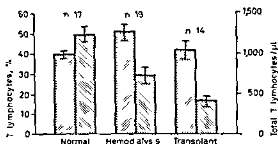


Fig 1 Percent T lymphocytes (dotted) and total T lymphocytes (hatched) per microliter of blood in normal subjects, hemodialysis patients and transplant recipients

³H thymidine (1 9 Ci/mM) and harvested after an additional 16–18 h of incubation Counts per minute (cpm) are presented after subtraction of control counts in cultures without mitogens

Results

The mean values of percent T lymphocytes in hemodialysis patients, allograft recipients and normal subjects were 51 3±4 5, 41 6±4 1 and 39 8±1 6, respectively (fig 1) The mean values of total T lymphocytes present in normal subjects, hemodialysis patients and allograft recipients were 1,241±82, 727±90 and 402±72/µl of blood, respectively (fig 1) Although the percentage of T lymphocytes was significantly ($p<0\ 05$) higher in the hemodialysis patients, the total number of T lymphocytes per microliter of blood was significantly lower ($p<0\ 001$) both in the hemodialysis patients and renal allograft recipients No association was apparent between sex, age, disease, duration of uremia and percent T lymphocytes or total T lymphocytes per microliter of blood

Data with regard to the correlation between percent T lymphocytes and the *in vitro* response to mitogens in the three categories of individuals are presented in table I Since all *in vitro* lymphocyte cultures possessed a constant number of lymphocytes (100,000) per tube with varying percentage of T cells, a correlation between the response to mitogens and the percent T lymphocytes in the preparation was thus determined The percentage of T lymphocytes in normal subjects was 39 8±1 6 with PHA and PWM response of 37,611±3,006 and 23,198±2,214, respectively As expected the correlation between percent T lymphocytes and

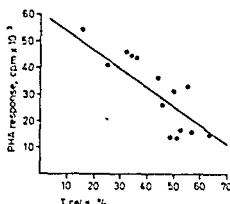


Fig 2 Correlation between percent T lymphocytes and *in vitro* response to PHA in transplant recipients r = -0 773, y = 60 273 14-701 676x

Table I Correlation between T cells and *in vitro* response to PHA and PWM

Category	Number of subjects	T cells, %	PHA cpm	PHA r	PWM cpm	PWM r
Normal	17	39 8±1 6 (30−51)	37,611±3,006 (20,890−67,950)	+0 427	23,198±2,214 (9,737−35,012)	−0 525[1]
Hemo-dialysis	19	51 3±4 5 (21−82)	34,782±2,660 (14,319−64,792)	+0 058	22,408±3,270 (11,009−55,372)	−0 083
Trans-plant	14	41 6±4 1 (16−64)	31,053±3,044 (13,723−54,417)	−0 773[2]	18,481±2,094 (8,333−34,315)	−0 829[2]

Value for percent cells and response to mitogens are presented as mean±SE. Figures in brackets indicate range

r=Correlation between the percent T cells in lymphocyte preparation and *in vitro* response to mitogens

[1] Significant at 5% level

[2] Significant at 1% level

the response of lymphocytes to PHA in the normal subjects was positive (r = +0 427) and that to PWM was negative and significant (r = -0 525). The percentage of T lymphocytes in hemodialysis patients was 51.3±4.5 with PHA and a PWM response of 34,782±2,660 and 22,408±3,270 No significant association was found between percent T lymphocytes and response to PHA (r = +0.058) or PWM (r = -0.083) The percentage of T lymphocytes in renal allograft recipients

was 41 6±33 with PHA and a PWM response of 31,053±3,049 and 18,481±2,084, respectively The correlation between T lymphocytes and response to both PHA (fig 2, r = –0 773) and PWM (r = –0 829) was highly significant but negative

Discussion

Hemodialysis patients, in spite of having a significantly higher percentage of T lymphocytes, had a significantly reduced number of total T lymphocytes as compared to normal subjects It has been emphasized recently by DELLON [4] that reporting rosettes as a percentage alone can be misleading Thus, both hemodialysis and renal allograft patients had significantly reduced numbers of total T lymphocytes Depression of certain parameters of cellular immunity such as delayed hypersensitivity [8, 11, 14] and *in vitro* lymphocyte response to VARIDASE [11] in hemodialysis patients may thus be related to the reduced number of circulating lymphocytes in these patients

As expected, in normal subjects T lymphocytes gave a positive correlation with response to PHA and a negative correlation with PWM (table I) However, in the renal allograft recipients percent T lymphocytes gave a highly significant but negative correlation with PHA response (table I, fig 2) In contrast, no correlation was observed between percent T rosettes and the response of lymphocytes to PHA and PWM in the hemodialysis patients The reason for the lack of correlation is not clear The use of ratios of stimulation rather than cpm did not improve the correlation Hemodialysis and renal allograft recipients often have increased number of blast like cells [3] The possibility that the Ficoll-Isopaque method of purification may alter the proportion and thus the functional properties of T cells by eliminating certain clones of cells is not ruled out Certain other factors such as the variation in the number of one or more subpopulations [5], surface properties or cell cycle phase of lymphocyte populations [13], and infection [16] may alter the qualitative and quantitative properties of T and B lymphocytes or their subpopulations

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Solubilisation von Gewebethromboplastin. Effekt verschiedener Detergentien[1]

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Key Words. Aminopeptidase · Blood coagulation · Brain thromboplastin · Detergents · Phospholipids

Abstract. The effect of 3 different detergents (Triton X 100, Lubrol WX and Nonidet) on brain thromboplastin was examined and compared with the solubilisation due to deoxycholate. The effect of deoxycholate is superior over that of the other examined detergents. Deoxycholate extracts thromboplastic activity from brain tissue to a large extent. Furthermore it has no interfering absorbance at 280 nm. The aminopeptidase of brain tissue may be separated from thromboplastin. It does not have any coagulation activity.

Durch frühere Untersuchungen wurde gezeigt, dass Gewebethromboplastin ein Protein-Phospholipid-Komplex ist, der durch organische Lösungsmittel (Pyridin) in seine beiden Hauptbestandteile gespalten werden kann. Nach Rekombination in Gegenwart des Lösungsmittels entfaltet der Komplex wiederum seine volle biologische Aktivität [2]. Da diese Aktivität an subzelluläre Partikel gebunden ist [2, 11], war eine weitere Reinigung des Proteinanteils nicht ohne weiteres möglich. Erst durch die Verwendung von Na-Desoxycholat [6] gelang es, Gewebethromboplastin löslich zu machen, die Aktivität aus dem Gewebe zu extrahieren und weitere Reinigungsschritte durchzuführen. Durch Gelfiltration in Gegenwart des Detergens war es möglich, das Protein vom Phospholipid auf einfache Weise zu trennen [7] und schliesslich rein darzustellen [1].

Bei der Präparation von Gewebethromboplastin waren DEUTSCH und IRSIGLER [3] auf eine Aminopeptidase gestossen und konnten nachweisen,

[1] Die Arbeit wurde mit Unterstützung des Österreichischen Forschungsrates, Projekt M2/2090 durchgeführt.

dass dieses Enzym nicht mit der Thromboplastinaktivität in Zusammenhang steht. In neuerer Zeit zogen PITLICK *et al.* [9] – in Analogie zur proteolytischen Wirksamkeit anderer Gerinnungsfaktoren – den Schluss, dass die Gerinnungsaktivität von Gewebethromboplastin auf der enzymatischen Aktivität des Proteins beruhe. Dies veranlasste uns, neuerlich den Einfluss der aus Gehirngewebe extrahierbaren Peptidase auf den Gerinnungsvorgang zu überprüfen. Da uns ferner keine Veröffentlichungen über die Wirkung anderer Detergentien als Desoxycholat (DOC) auf Gewebethromboplastin (GT) bekannt sind, soll im folgenden der Effekt von 3 verschiedenen Detergentien dargestellt und mit der DOC-Wirkung verglichen werden.

Material und Methoden

Gewebethromboplastin wurde wie früher beschrieben [2] aus menschlichem Gehirn hergestellt, nur wurde entsprechend den Ergebnissen von HVATUM und ERYDZ [6] graue und weisse Gehirnsubstanz verwendet. Unter GT verstehen wir im folgenden das mehrfach durch Ultrazentrifugation gereinigte Gehirngewebe. Ungereinigtes GT ist die Gehirnpräparation, die aus dem Azetonpulver durch Extraktion und Zentrifugation bei 1000 g gewonnen wird. Die GT-Suspension wurde so eingestellt, dass sie durch weitere Verdünnung nicht aktiver wurde. Die Prüfung der Aktivität der einzelnen Präparationen erfolgte wie früher beschrieben [2]. Die Solubilisation von GT wurde in Anlehnung an HVATUM und ERYDZ [6] durchgeführt. Zu dem aus 46 ml ungereinigtem GT durch Zentrifugation bei 105 000 g gewonnenen Sediment wurde 11,5 ml 0,25%ige Detergenslösung zugesetzt. Anschliessend erfolgte eine neuerliche Zentrifugation bei 105 000 g während 2 h. Das Detergens wurde aus dem so gewonnenen Überstand durch 48- bis 72stündige Dialyse entfernt. Dialysiert wurde gegen 4mal bis 6mal 2 Liter Veronalpuffer pH 7,4. In Vorversuchen war festgestellt worden, dass diese Dauerdialyse ausreicht, um die Detergentien soweit zu entfernen, dass sie den Gerinnungsvorgang nicht mehr hemmen. An Detergentien wurde Na-Desoxycholat (Merck), Triton X-100, Lubrol WX (Sigma) und Nonidet (Shell) verwendet. Die Peptidaseaktivität wurde wie andernorts beschrieben [3] bestimmt. Als Substrate verwendeten wir Alanyl-β-naphthylamid und Lysyl-β-naphthylamid (Serva). Die qualitative Analyse der Phospholipide erfolgte nach Extraktion der lyophilisierten Präparationen nach FOLCH *et al.* [5] dünnschichtchromatographisch an Merck-DC-Platten. Laufmittel: Chloroform-Methanol/Wasser 64:25:4 (v/v). Anfärbung der Fraktionen mit dem Reagens nach DITTMER und LESTER [4].

Resultate

In Tabelle I sind die Peptidaseaktivitäten dargestellt, wie sie in den einzelnen Stufen der GT-Reinigung auftreten. Aus Tabelle I ist ersicht-

Tabelle I Peptidase und Gerinnungsaktivität von verschiedenen Präparationsstufen im Zuge der Gehirnthromboplastinreinigung

Gehirnpräparation	Peptidaseaktivitat, nmol/ml h		Gerinnungsaktivität, arbitrary units[1], %
	Substrat		
	Alanyl β naphtylamid	Lysyl β naphtylamid	
Kochsalzextrakt, 0,4 g/ml	5670	3911	–
Azetonpulver, 0,4 g/ml	606	418	–
Thromboplastin, 1mal bei 100000 *g* zentrifugiert	168	55	100
Thromboplastin, 3mal bei 100000 *g* zentrifugiert, lyophilisiert	0	0	100
1 Überstand bei der Thromboplastinreinigung, 100000 *g*	553	223	24
3 Überstand bei der Thromboplastinreinigung, 100000 *g*	87	36	<1

[1] Arbitrary units = Gerinnungsaktivität von ungereinigtem GT wurde willkürlich als 100% angenommen

lich, dass das Enzym eine hohere Affinitat zu Alanyl-β-naphtylamid als zu Lysyl β-naphtylamid hat, wie dies auch von Pitlick *et al* [9] gefunden wurde Ferner sieht man, dass das Enzym wasserloslich ist, da es beim Zentrifugieren im Überstand erscheint, während die Aktivitat im Sediment mit der Zahl der Zentrifugationsschritte laufend abnimmt Nach 3maligem Waschen bei 100 000 *g* wurde im Sediment keine Aktivitat *mehr nachgewiesen Die thromboplastische Aktivitat war* dabei im Sediment voll erhalten Im Überstand nimmt die thromboplastische Aktivitat mit der Zahl der Zentrifugationsschritte dabei laufend ab

Bei dem Versuch, die thromboplastische Aktivitat aus dem Sediment mit Hilfe von Detergentien in Losung zu bringen (Tab II), zeigt sich die deutliche Überlegenheit von DOC Obwohl Triton X-100 eine grossere Eiweissmenge extrahiert, zeigt das dialysierte Extrakt eine deutlich langere Rekalzifizierungszeit Der Solubilisierungseffekt der beiden anderen getesteten Detergentien ist noch geringer

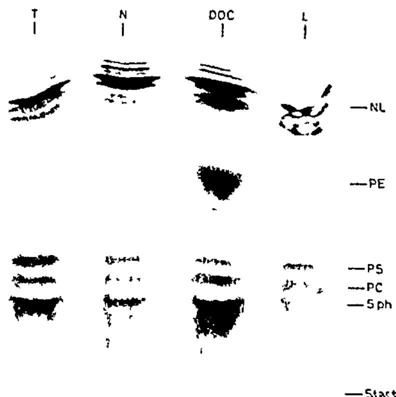


Abb 1 Dünnschichtchromatographische Auftrennung der Phospholipide von mittels verschiedener Detergentien gewonnenen GT-Präparationen Laufmittel Chloroform/Methanol/Wasser 64 25 4 Sprühmittel Dittmer-Lester Reagens T = Triton X 100 N = Nonidet DOC = Na Desoxycholat L = Lubrol WX NL = Neutrallipide PE = Phosphatidyläthanolamin PS = Phosphatidylserin PC = Phosphatidylcholin Sph = Sphingomyelin

Tabelle II Effekt verschiedener Detergentien auf die Solubilisation von Gehirnthromboplastin *Die spezifische Aktivität von DOC-Extrakt wurde willkürlich mit 100% angenommen* (ungereinigtes GT 12,9 sec)

Detergens, 0,25%	*Zeit*, sec	Spezifische Aktivität, %
DOC	13,0	100
Triton X-100	24,3	1,7
Lubrol WX	77,3	0,1
Nonidet	45,0	0,4

In Abbildung 1 ist im Dünnschichtchromatogramm die Phospholipidzusammensetzung des mit verschiedenen Detergentien extrahierten GT zu erkennen. Während Lubrol WX kein aktives Phospholipid extrahiert, findet sich im Triton 100-X- und Nonidet-Extrakt bloss eine schwache Phosphatidyläthanolaminbande. Lediglich DOC extrahiert Phosphatidylcholin und Phosphatidyläthanolamin in höherem Masse.

Diskussion

Obwohl früher bereits [3] nachgewiesen werden konnte, dass die aus Gehirn extrahierbare Aminopeptidase am Gerinnungsvorgang nicht beteiligt ist, wurde von PITLICK *et al.* [9] ein enger Zusammenhang zwischen proteolytischer Enzymaktivität von GT und der Gerinnungsaktivität angenommen. BJØRKLID *et al.* [1] untersuchten dieses Problem in neuerer Zeit und konnten ebenso wie wir in gereinigten GT-Präparationen keine Peptidaseaktivität finden. Auch eine Rekombination des gereinigten Proteinanteils mit Phospholipiden und der Zusatz von Co^{++}-Ionen liess keine messbare Peptidaseaktivität auftreten. Dies ist in guter Übereinstimmung mit unseren andernorts beschriebenen Versuchen [3], in denen gezeigt werden konnte, dass durch Rekombination des Proteinanteils mit Phospholipiden wohl die thromboplastische, nicht jedoch die Peptidaseaktivität rekonstituiert werden kann. Auch der Einfluss von verschiedenen Ionen auf die Peptidase war schon früher von uns untersucht worden [3]. Die völlige Unabhängigkeit von Peptidase- und thromboplastischer Aktivität wird durch die hier durchgeführten Versuche neuerlich bestätigt. Es gelingt, die Peptidase durch ausreichendes Waschen in der Ultrazentrifuge von GT zu trennen, so dass im Sediment nur mehr die thromboplastische, im Überstand nur noch die proteolytische Aktivität vorhanden ist.

HVATUM und PRYDZ [6] bedienten sich als erste der Eigenschaft von Detergentien, Protein-Phospholipid Komplexe in Lösung zu bringen. Sie verwendeten Na-Desoxycholat. Es gelang ihnen so, die thromboplastische Aktivität quantitativ in Lösung zu bringen. Daneben war es ihnen möglich, durch Gelfiltration in Gegenwart des Detergens den Proteinanteil vom Phospholipidanteil des GT zu trennen [7]. Nun war der Weg zur weiteren Reinigung des Proteinanteiles offen. BJØRKLID *et al.* [1] konnten das Protein soweit reinigen, dass in der Polyacrylamidelektrophorese nur mehr eine Bande nachweisbar war. Da uns ein Vergleich der Wirkung

von DOC mit anderen Detergentien nicht bekannt war, testeten wir 3 weitere Detergentien auf ihre Fähigkeit, thromboplastische Aktivität in Lösung zu bringen. Als häufig zur Extraktion ribosomaler Proteine verwendetes Detergens untersuchten wir die Wirkung von Triton X-100. Das zweite verwendete Detergens, Lubrol WX, wurde als stärkeres Extraktionsmittel für mikrosomale Enzyme als Triton X-100 beschrieben [10]. Schliesslich untersuchten wir noch die Wirkung von Nonidet, einem zur Extraktion von DNA-Polymerasen verwendeten Detergens [8]. Die Konzentration der einzelnen Detergentien wurde, nach hier nicht gesondert dargestellten Vorversuchen, der von HVATUM und PRYDZ [6] für DOC gefundenen optimalen Konzentration von 0,25% angeglichen. Obwohl nach dem Entfernen des Detergens durch Dialyse in jedem Falle wieder ein trübe Suspension entstand und sich nach 1- bis 2maligem Einfrieren ein Bodensatz bildete, wie dies für DOC-behandeltes GT typisch ist, war keines der untersuchten Detergentien imstande, thromboplastische Aktivität in einem dem DOC vergleichbaren Ausmass in Lösung zu bringen. Dies findet seine Erklärung durch unsere dünnschichtchromatographischen Untersuchungen des Phospholipidanteiles. Nach Extraktion mit Lubrol kann keine aktive Phospholipidbande nachgewiesen werden. Triton X-100 und Nonidet extrahieren etwas Phosphatidylathanolamin, während im DOC-Extrakt sowohl Phosphatidylcholin als auch Phosphatidylathanolamin nachgewiesen werden können. Nur in diesem Falle sind also Protein- und Phospholipidanteil in einem Verhältnis vorhanden, das für die thromboplastische Aktivität notwendig ist. Zusätzlich dazu haben noch zwei der genannten Substanzen – Triton X-100 und Nonidet – den Nachteil, dass sie bei 280 nm eine hohe Eigenabsorption zeigen, ein Nachteil, der sich bei der zur weiteren Reinigung notwendigen Gelchromatographie in Gegenwart des Detergens auswirkt.

Zusammenfassung

Der Effekt von 3 verschiedenen Detergentien (Triton X 100, Lubrol WX und Nonidet) auf Gehirnthromboplastin wurde untersucht und mit der Solubilisation durch Desoxycholat (DOC) verglichen. Es ergab sich eine eindeutige Überlegenheit von DOC, welches die thromboplastische Aktivität optimal aus Gehirngewebe extrahiert und keine störende Eigenabsorption bei 280 nm aufweist. Ferner wurde neuerlich gezeigt, dass die aus Gehirngewebe extrahierbare Aminopeptidase von der thromboplastischen Aktivität trennbar ist und selbst keine gerinnungsfördernden Eigenschaften aufweist.

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Haemoglobin D and D Thalassaemia

A Family Report, Comprising 18 Members

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Key Words D thalassaemia Haemoglobin D Haemoglobinopathies Thalassaemia

Abstract On the occasion of a double heterozygote case of D haemoglobinopathy/β thalassaemia (D thalassaemia) from Epirus (Greece) a family study was performed It comprised 18 members belonging to 3 generations and revealed the presence of an additional D thalassaemia case 4 D haemoglobinopathy heterozygotes 5 β thalassaemia heterozygotes and 7 normal persons The D thalassaemia cases were initially considered as Hb D homozygotes according to their electrophoretic phenotype the family study however showed the true nature of their stigmata These patients presented with mild jaundice splenomegaly and moderate anaemia while the Hb D heterozygotes were asymptomatic

D haemoglobinopathy is a relatively rare condition It was first described by ITANO [12] in 1951 Since then sporadic cases have been reported in various parts of the world such as Algeria [4] England [18] Turkey [1] USA [5 16] Portugal [2] India [3] and Greece [10] Most of the cases presented as double heterozygotes of Hb D with other haemoglobin mutations Double heterozygosity of Hb D/β thalassaemia has rarely been described namely in a Persian girl by HYNES and LEHMANN [11] in 1956 in 4 persons from Bombay by SUKUMARAN *et al* [19] in 1960 and in 3 persons from Greece by DELIYANNIS *et al* [7] in 1969 All these patients presented with mild clinical manifestations such as slight hepato-splenomegaly and jaundiced sclerae they had an otherwise normal body habitus and a good physical condition

Methods

Haemoglobin, haematocrit (PCV) and red cell (RBC) measurements were performed by the cyanmethaemoglobin, microhaematocrit and celloscope counter, respectively Bone marrow examination, reticulocyte count, serum iron and bilirubin levels were also performed Thin blood films, stained with May Grünwald Giemsa were inspected for red cell morphology Osmotic fragility, sickling and solubility tests [13] were made (table I) G-6-PD was also measured in 4 siblings

Haemolysates were prepared as was previously described [20]

Determination of Hb F was made by the method of Singer *et al*, as modified by Fisher [8] and by electrophoresis in acid agar, pH 5.9 [21] The latter helped in distinguishing Hb D from Hb S [13] Haemoglobin electrophoresis was also performed in cellulose acetate starch gel, pH 8 6, starch block for the elusion of Hb A_2 and in paper

For quantitations of haemoglobins A and D, electrophoresis on cellulose acetate strips was performed The haemoglobin bands were excised and eluted with distilled water After adjustment to an appropriate volume, the haemoglobin concentrations were estimated spectrophotometrically at 415 nm

A blood sample from the initial case (propositus) was sent to MRC Abnormal Haemoglobin Unit, University of Cambridge (England) for the determination of the exact nature of Hb D under investigation

Case Report and Family Study

The propositus a fairly well-developed 24-year-old soldier from Epirus (Greece), was admitted at 424 Army General Hospital of Thessaloniki on August 9, 1973, because of pleurisy (tuberculous) and recurrent episodes of mild jaundice with splenomegaly A summary of the haematologic study is shown in table II (case No II-6) PCV 43%, Hb 13 9 g%, RBC $6 1\times10^6$, MCV 70 μm^3, MCH 23 pg

Table I Solubility test

Phosphate buffer solutions	Solubility depending on the type of haemoglobin, %				
	Hb A	Hb AD[1]	Hb D[2]	Hb AS	Hb SS
1 10 M	100	100	100	100	100
2.49 M	85	85	85	25	8
2.87 M	20	20	20	10	2

[1] Hb AD of the father
[2] Hb D of the propositus

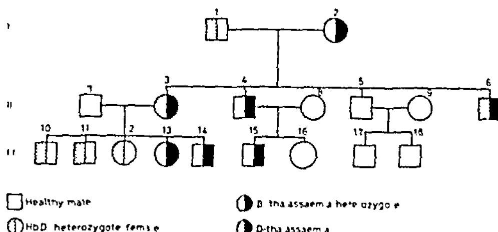


Fig 1 Pedigree containing Hb D heterozygotes β thalassaemia heterozygotes Hb D/β thalassaemia double heterozygotes and healthy members in 3 generations

Table II Main haematologic findings of the whole family in 3 generations

Case No in fig 1	Age years	Red cells $\times 10^6/\mu l$	Htc %	Hb g%	MCV μ^3	MCHC %	Hb A_2 %	Hb F %	Hb D %	Diagnosis
I 1	70	4 6	41	13 9	89	34	4 2	0 5	48 2	Hb D heterozygous
I 2	58	4 0	32	10 8	80	33	3 7	1 4	–	β thalassaemia heter
II 3	35	4 5	32	11 2	71	35	5 3	6 0	88 7	D thalassaemia
II–4	28	5 2	35	12 0	67	34	5 9	2 5		β-thalassaemia heter
II 5	26	5 0	46	14 8	90	32	2 8	0 7		normal
II 6	24	6 1	43	13 9	70	32	5 1	2 5	92 4	D thalassaemia propositus
II 7	40	4 5	42	14 1	93	33	3 1	0 9		normal
II 8	25	4 0	37	12 6	92	34	2 4	1 0		normal
II 9	23	4 3	42	12.8	97	30	3 0	0 9		iron deficiency
III 10	11	4 9	41	13 0	83	31	4 3	0 6	49 0	Hb D heterozygous
III 11	9	4 7	40	12.9	85	32	4 4	0 7	48 3	Hb D heterozygous
III 12	7	4 9	41	13 2	83	32	4 8	1 4	48 8	Hb D heterozygous
III 13	6	4 8	34	10 8	71	32	4 5	1 7		β thalassaemia heter
III 14	5	4 8	38	11 9	73	31	5 3	1 8		β-thalassaemia heter
III 15	4	4 7	38	12 0	80	31	6 6	2 1		β thalassaemia heter
III 16	2	3 8	35	11 2	92	32	2.6	1 2		normal
III 17	4	4 2	35	10 4	83	30	3 0	0 5		iron deficiency
III 18	1	4 3	37	12 9	88	33	2 3	1 0		normal

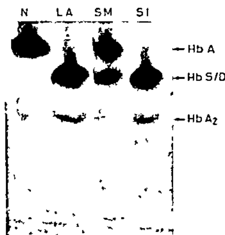


Fig 2 Electrophoretic diagram showing normal Hb (N), Hb of propositus (L. A) a heterozygous sickler (S M), and propositus' sister (S I)

MCHC 32% and WBC 7,000/mm³ The differential count was within normal limits Red cell morphology was characterized by microcytosis, numerous target cells and relative hypochromia Reticulocyte count 2 7% Red cell fragility to hypotonic saline solutions was decreased Serum iron 142 μg% Bilirubin (indirectly reacting) ranged between 3 and 7 mg% G-6-PD activity was within normal limits Total serum protein level 7 g%, with 4 4% albumin and 2 6% globulins The protein electrophoretic pattern was also within normal limits On X ray no skeletal abnormalities were present Bone marrow specimens showed a marked hyperplasia of red cell precursors

Haemoglobin electrophoresis run simultaneously with Hb S-A and Hb A in alkaline pH, revealed a homozygous Hb S pattern with increase in Hb A_2 (fig 2) As the sickling test was repeatedly negative, a repeat electrophoresis in acid agar was also performed In this case the haemoglobin under investigation had the same mobility as Hb A while Hb S (run at the same time) occupied the place of Hb A_2 in the electrophoretic diagram (fig 3) Furthermore, the Hb A_2 was found to be 5 1%, using the starch block electrophoresis and elution technique [21] Alkali resistant Hb was 2.5% Finally, the differential solubility test showed normal solubility for Hb A and Hb D and decreased solubility for Hb S S and Hb S-A (table I)

In view of the above findings, suggestive of Hb D homozygosity, a family study was performed, in order to document genetically the haemoglobin under investigation The propositus family consisted of 18 members, his parents, aged 70 and 58 one sister aged 35 and two brothers, aged 28 and 26, respectively, along with their mates and children His sister had 5 children while his brothers had two each The family data are presented in table II and in figure 1 This investigation affords evi-

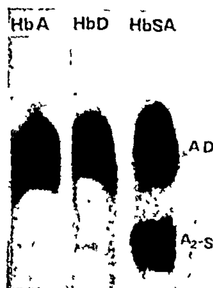


Fig 3 Haemoglobin electrophoresis in agar gel, pH 5.9 From left normal propositus, and heterozygous sickler

dence that the case under investigation was a double heterozygote Hb D β thalassaemia (fig 2, 3)

In addition, the MRC Abnormal Haemoglobins Unit University of Cambridge (England) identified the abnormal haemoglobin as Hb D_{Punjab}. We would like to express our gratitude to Dr H LEHMANN and Dr R STATHOPOULOU for their invaluable contribution to our investigation

Discussion

Homogyzous D-haemoglobinopathy is clinically and haematologically similar to double heterozygous Hb D/β-thalassaemia (D thalassaemia) Both present with symptoms and signs of a mild haemolytic anaemia Electrophoretically, two main fractions are present that of Hb A_2 and another haemoglobin in the place of Hb S [6, 21] It should be stated, however, that the Hb A_2 fraction is increased in the cases of D thalassaemia (fig 2, 3) The latter was noted in one out of the 4 cases described by SUKUMARAN *et al* [19]

The propositus (II-6) was initially considered as a Hb D homozygote, due to the fact that only Hb A_2 and Hb D were present on electrophores-

is. The family study, however, revealed that his mother (I-2), had typical heterozygous β-thalassaemia, while his father (I-1), was a Hb D heterozygote, with his Hb A_2 fraction increased (4 2%) and equal parts of Hb A and Hb D A possible increase of Hb A_2 in heterozygous Hb D state has been suggested by LEHMANN [15] In that case, Hb A was present in a greater proportion, compared to Hb D (Hb A > Hb D)

The above described parents gave birth to 4 apparently normal siblings Two of them, the propositus and his sister (II-3), had D thalassaemia with an electrophoretic phenotype of homozygous D haemoglobinopathy (fig 2) Here the interaction of the abnormal genes resulted in the disappearance of Hb A It has to be taken that the mother of the propositus should have one β^{0}-gene [9] This woman was married to a Hb D heterozygote man and gave birth to double heterozygote offsprings It should be again noted that both our D thalassaemia cases (II-3 and II 6) had mild symptoms and signs of anaemia and splenomegaly and showed mainly Hb A_2 and Hb D on electrophoresis The Hb A_2 fraction was increased in both (5 1 and 5 3%, respectively), while the alkali resistant Hb F was 2 5 and 6 0%, respectively Increased amounts of Hb F were reported by HYNES and LEHMANN [11] in their case NECHELES *et al* [17] believe that Hb F should be increased in such cases, exceeding 5% Among the 4 cases described by SUKUMARAN *et al* [19], only one had increased Hb A_2 and another had an insignificant increase in Hb F (1 7%)

The interaction of genes, which was postulated in our cases, was not observed in the case of D thalassaemia described by LEE and HUISMAN [14] This was due to the combination of heterozygous β-thalassaemia and Hb $D_{St\ Louis}$ which is an α-chain mutation Of the two brothers of the propositus, one (II-4) was a β-thalassaemia heterozygote and the other (II-5) was normal The above, married to normal women, had two children each One of the children of II-4, the son (III 15), was found to be a β-thalassaemia heterozygote, just as his father (Hb A_2 6 6%), the rest were normal

The most interesting member of the propositus family, however, was his sister (II-3), the other double heterozygous (D thalassaemia) case She was married to a normal man and had 5 children, to whom she distributed her stigmata Three of them were found to be Hb D heterozygotes, and the other two β-thalassaemia heterozygotes (fig 1, table II) As in the case of their mother-grandfather, the Hb D heterozygous grandchildren had increased Hb A_2 (4 3, 4 4 and 4 8%, respectively) and almost equal proportions of Hb D and Hb A

In conclusion, the complete pedigree haematologic study of the 3 generations of this family, which revealed the presence of both Hb D and β-thalassaemia genes, except for identifying the exact nature of the haemoglobinopathy under investigation, gave the opportunity for some interesting genetic considerations The origin of the family from the same area of Northern Greece (Epirus), as that of the cases described by DELIYANNIS *et al* [7], probably suggests an increased incidence of Hb D in this area

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Hemoglobin Beograd ($\alpha_2\beta_2$ 121 Glu→Val) Interacting with β-Thalassemia[1]

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Key Words. Hemoglobin Beograd · Hemolytic anemia · Thalassemia

Abstract. Hematological and biochemical findings in a family with hemoglobin (Hb) Beograd interacting with β-thalassemia are presented. Hb Beograd ($\alpha_2\beta_2$ 121 Glu→Val) was found in 3 members. In two members it interacted with β-thalassemia. These two double heterozygotes had anemia of intermediate severity and splenomegaly. Studies with ^{51}Cr and ^{59}Fe showed a shortened life span of red cells and ineffective erythropoiesis. The abnormal Hb amounted to 86–87% and Hb F to 5–7%. No Hb A was present. One member of the family was heterozygous for Hb Beograd. He showed normal clinical and hematological findings. The abnormal hemoglobin was 39%. Four members of the family were heterozygotes for β-thalassemia. The interaction between β-thalassemia and β-chain variants is discussed.

Thalassemia is a relatively common disease in Yugoslavia. Although there is no exact figure on the incidence of thalassemia in this country it has been estimated that about 1% of Yugoslavian population carries thalassemia gene [4]. Fraser *et al.* [7] found an incidence of thalassemia of 7.1% among the population of Dalmatian coast and SR Macedonia. A more exact figure was obtained by [illegible] *et al.* [12] who examined 2,770 school children from SR Macedonia and found an incidence of β-thalassemia of 4.7%. Abnormal hemoglobins have also been found in Yugoslavia. The most common is Hb Lepore. This variant has been found in homozygous and heterozygous state, and also in combination with β-thalassemia [5].

[1] This work was financed in part by NIH PL-480 program ([illegible])

Recently we reported finding of a new abnormal hemoglobin termed Hb Beograd or $\alpha_2\beta_2$ 121 Glu→Val [5]. In this paper we present the clinical and laboratory findings in this family where Hb Beograd interacts with β-thalassemia

Methods

Standard hematological methods were used to determine hemoglobin, hematocrit, red cell and reticulocyte count, bone marrow examination, determination of serum iron, total iron binding capacity and bilirubin

Red cell hemolyzates were studied by starch block electrophoresis in barbital buffer, pH 8 8 [8], and by starch gel electrophoresis in Tris-EDTA boric acid buffer, ph 9 0 [6] The amount of hemoglobins F, A_2, A and the abnormal hemoglobin were determined by starch block electrophoresis and by chromatography on columns of DEAE Sephadex [2, 9] Hemoglobin F was also determined by alkali denaturation [1]

Case Reports

Case 1 The proposita (III, 2 in fig 1) was a 24-year-old female from the area around Beograd when she was seen for the first time at the Internal Clinic B during 1961 She asked for medical care because of tiredness paleness, anorexia and abdominal pain which appeared in 1960, immediately after she gave birth to her first child However, the patient has noticed tiredness since her childhood During 1957 she was examined by a local physician and was told to suffer from anemia She got her first menses when she was 19 years old

She had an enlarged spleen (6 cm below the costal margin) of increased consistency The liver was palpable 4 cm below the costal margin She was pale with mongoloid facies and subicteric sclerae Hematologic examination revealed mild anemia with anisopoikilocytosis, hypochromia and many target cells Hb 9 6 g%, RBC 3 8 million/μl reticulocytes 10%, Bilirubin 2 1 mg% Increased osmotic fragility (beginning of hemolysis in 0 35% NaCl, complete hemolysis in 0 15% NaCl), serum iron 158 μg% transferrin saturation 63% The bone marrow showed 72% erythroblasts and 73% sideroblasts (normal 25–70%) and also extracellular iron Fetal hemoglobin determined by alkali denaturation was 9%

Studies by ^{51}Cr showed a shortened life span (T_{50} 15 days) The result of ferrokinetic studies are shown in figure 2 ^{59}Fe clearance was rapid with early plateau' formation, the erythrocyte ^{59}Fe incorporation curve show a slow rise with a reduced maximum level (42%) The surface counting revealed a rapid rise and high activity over the sacrum and a secondary rise of activity over the spleen

Case 2 The brother of case 1 (III, 3 in fig 1) a manual worker, 21 years old, was admitted to the Internal Clinic B in 1965, because of paleness and abdominal pain On physical examination he was found to be pale, subicteric with enlarged spleen (4 cm below the costal margin) Hematologic examination revealed mild ane

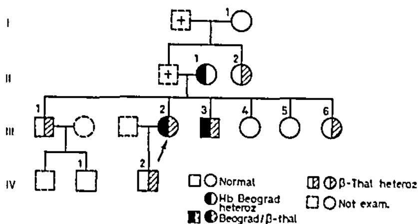


Fig. 1. Family tree The arrow indicates the proposita

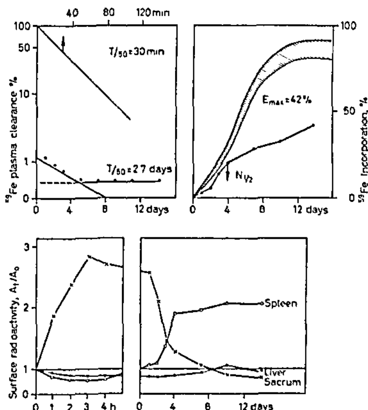


Fig 2 Ferrokinetic studies in the proposita ^{59}Fe incorporation, hatched area = normal values

Table I Clinical and laboratory data

Subject (see fig 1)		Age, years	Icterus	Spleno-megaly	Bilirubin mg%	Hb g%	RBC ×10⁶/µl	Ret %	Aniso-cytosis	Poikilo-cytosis	Hypo-chromia	Target cells	Hemoglobin, %		
													F	A_2	Beograd
I	1	76	–	–		12 8	4 0	0 4	–	–	–	–	0 9	2 7	–
II	1	56	–	–	0 55	12 1	4 0	1 0	–	–	–	–	0 7	2 2	38 0
	2	40	–	–	0 41	8 4	3 2	5 0	+	+	+	+	1 0	5 4	–
III	1	35	+	+	1 3	12 8	4 2	2 2	+	+	+	+	1 6	5 5	–
	2	30	++	++	2 1	9 6	3 7	9 0	++	++	++	++	6 5	7 4	87 1
	3	23	++	++	1 75	10 8	3 7	8 4	++	++	++	++	3 0	5 2	86 0
	4	27	–	–	0 5	9 5	3 3	0 4	+	+	++	–	0 7	2 4	–
	5	32	–	–		12 7	4 2	0 2	–	–	–	–	0 6	2 7	–
	6	21	+	±	1 5	10 8	3 9	2 6	+	+	+	+	1 2	5 4	–
IV	1	9	–	–									1 4	2 6	–
	2	7	–	±	0 65	10 0	3 8	2 6	+	+	+	+	3 0	5 6	–

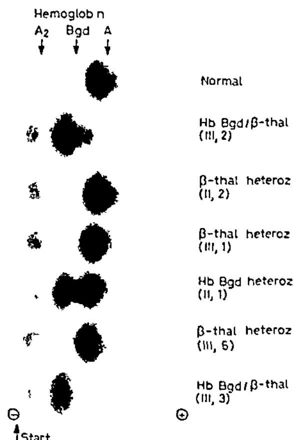


Fig 3 Starch block electrophoresis in veronal buffer, pH 8 8

mia with the typical abnormalities of thalassemia intermedia Hb 10 8 g%, RBC 3 8 million/µl reticulocytes 8 4% Bilirubin 1 7 mg% Increased osmotic fragility, serum iron 210 µg%, transferrin saturation 100% In the bone marrow there were 73% erythroblasts and 92% sideroblasts, extracellular iron was also present

The results of ferrokinetic studies were similar to those found in his sister (subject III, 2 in fig 1) ^{59}Fe clearance was rapid (37 min) incorporation in erythrocytes reduced (61%), on surface counting initial rapid rise over the sacrum and later, high activity over the spleen

At that time diagnosis of thalassemia minor was made

Family study During 1967 a family study was made The pedigree of this family is shown in figure 1 Of the 11 subjects examined, one was carrier of an abnormal hemoglobin later identified as Hb Beograd [5] 4 displayed the thalassemia trait and 2 presented Hb Beograd/β thalassemia double heterozygosis The starch block electrophoretic patterns of hemolyzates of some members of this family are shown in figure 3

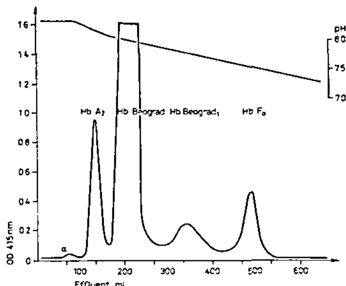


Fig 4 DEAE Sephadex chromatography of hemolyzate of the proposita with Hb Beograd/β thalassemia (III,2)

The clinical and laboratory data of all examined members of the family are shown in table I The presence of Hb Beograd was silent both clinically and hematologically The amount of Hb Beograd in the single heterozygote was 38% Subjects II 1 III 1 III 6 and IV,2 showed typical findings for β thalassemia trait. Although the father of cases 1 and 2 could not be examined he was most likely heterozygote for thalassemia since his sister (II,2) is carrier of β thalassemia. The two patients (III,2 and III,3) with Hb Beograd and β thalassemia showed findings of Cooley's anemia of intermediate severity Separation and quantitation of the abnormal hemoglobin by column chromatography on DEAE Sephadex showed absence of Hb A in these two subjects (fig 4) The abnormal hemoglobin amounted to 86 and 87% in patients III,2 and III,3 respectively

Discussion

Hb Beograd ($\alpha_2\beta_2$ 121 Glu→Val) is a β-chain variant with a substitution of glutamyl to valyl residue in position 121 [5] Since the residue in position 121 does not participate in contacts with heme or between chains, no disturbance of the functional and physical properties of the ab-

normal molecules is expected Apparently there is also no interference by these substitutions with bonding by other neighboring amino acid residues

A single heterozygote for Hb Beograd found in this family was both clinically and hematologically normal The two patients with Hb Beograd and β thalassemia, however, showed a moderately severe anemia and splenomegaly, the picture resembling Cooley s anemia of intermediate severity

The results of erythrokinetic studies are identical with those previously obtained in thalassemia [10, 11] Shortened red cell survival indicates an increased hemolysis ^{59}Fe studies suggest a moderately ineffective erythropoiesis (slow rise and reduced maximum of erythrocyte incorporation curve, high activity over the sacrum) with increased erythrocyte destruction in the spleen (high secondary activity over the spleen) Patient III, 2 was splenectomized in 1966 in another institution because of wrong diag nosis Her health has been improved although the hematologic findings did not show any difference from those observed before splenectomy

The action of the thalassemia gene in our two patients heterozygous for both β thalassemia and Hb Beograd appears to be complete reduction in the rate of synthesis of the normal β chains Hemoglobin A was completely absent indicating that the β thalassemia gene is of 'non hemoglob in A producing type, i e of the β^0-type Hb Beograd was present in amounts of 86–87% of the total the remainder consisting of hemoglobins F and A_2

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Trisomy 11 in Acute Phase of Chronic Myeloid Leukemia

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Key Words Chromosome aberrations Chromosome banding Karyotype Leukemic cells Myeloid leukemia Trisomy 11

Abstract A female patient with chronic myeloid leukemia in acute phase was found to have trisomy 11 in her bone marrow cells The contemporary existence of different clones suggests the following clonal evolution 46 XX→46 XX Ph→47 XX Ph +11→48 XX Ph,+11 +Ph It is suggested that the cytogenetic events leading to trisomy C in advanced cases of chronic myeloid leukemia are of a different character than those which often results in trisomy C in other myeloid disorders

The banding techniques have made it possible to recognize each human chromosome pair Thus bone marrow chromosomes from patients with hematological disorders can now be examined with better tools in order to evaluate the degree of specificity or possible pathogenetic or prognostic significance of abnormalities

Trisomy C is one of the most frequently reported chromosomal abnormalities in myeloid disorders Until now, 20 cases of trisomy C have been investigated by means of the banding methods [1–9 11, 12, 14] These cases have all involved chromosome No 8 or 9 with a single exception one case of chronic myeloid leukemia (CML) in acute phase in which trisomy of No 10 was found [1] We report here another case of myeloid disorder with trisomy C involving a chromosome pair not previously reported in this kind of anomalies

Case Report

The patient was a 74 year old woman who after 5 months of fatigue loss of weight and hyperhidrosis was admitted on September 18 1973 Neither hepatome

galy, splenomegaly or enlarged lymph nodes were found Hemoglobin 9 9 g/100 ml, reticulocyte count 1 8%, platelet count $287\times10^3/\mu l$, WBC $52\times10^3/\mu l$ with 11% myeloblasts, 7% promyelocytes, 28% myelocytes, 1% metamyelocytes, 16% band forms 12% neutrophils, 1% eosinophils and 24% lymphocytes 2% of the peripheral nucleated cells were erythroblasts The bone marrow was markedly hypercellular with a distribution of myeloid cells resembling that of the peripheral blood As treatment 100 mg Purinethol® (Wellcome) per day was given The differential count was normalized The bone marrow became hypocellular, and blood transfusions were needed The WBC fell to $1\text{–}1.5\times10^3/\mu l$ The platelet count fell to $10\text{–}20\times10^3/\mu l$ Jaundice developed and within a few days the condition deteriorated and the patient succumbed from septic shock (*Pseudomonas aeruginosa*) on November 24, 1973 Autopsy revealed a slight splenomegaly (spleen weight 300 g) and disseminated hemorrhages

Methods

Bone marrow samples for chromosomal investigation were obtained 3 weeks after initiation of chemotherapy The chromosomal analysis was performed by direct preparation of bone marrow cells using a slight modification of the trypsin-Giemsa method described by SEABRIGHT [13]

Cytogenetic Findings

43 mitoses were analysed The results are shown in table I The chromatin was quite blurred, but successful banding was obtained in five mitoses of the 48-type and the extra C chromosome could easily be recognized as a No 11 (fig 1) The two philadelphia chromosomes were derived from pair No 22 The preparations were not of such a quality that the eventual translocations of the deleted parts could be recognized Also two 46,XX mitoses and one 46,XX,Ph were banded

Table I Chromosomal findings in bone marrow cells

Number of mitoses	Karyotype
6	46,XX
12	46,XX,Ph
2	47,XX,Ph',+C
23	48,XX,Ph',+C,+Ph'

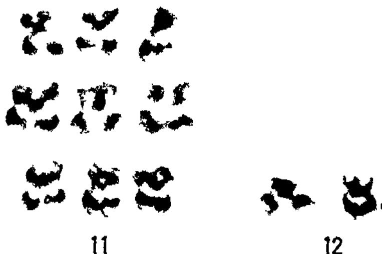


Fig 1 Partial karyogram of three mitoses showing trisomy 11 Chromosome No 11 can be clearly distinguished from No 12

Discussion

From a clinical point of view this case could be either differentiating acute leukemia or acute phase of CML The distribution of the chromosomal abnormalities, however, suggests a clonal evolution 46,XX→46,XX,Ph'→47,XX,Ph',+11→48,XX,Ph',+11,+Ph' This strongly favours the latter interpretation

The identification of the supernumerary group C chromosome as a No 11 is of interest The earlier investigated cases of 8- or 9-trisomy are distributed as follows using the authors designation of diagnoses acute myeloblastic leukemia (6 cases) [4, 7, 9], acute myelomonoblastic leukemia (1 case) [12], blast crisis of CML (2 cases) [5], myeloproliferative disorder (2 cases) [8, 14], sideroachrestic anemia (1 case) [7], sideroblastic anemia (1 case) [6], pancytopenia (2 cases) [2], myelosclerosis (2 cases) [3], essential thrombocytosis (1 case) [11] and polycythemia vera (1 case) [11] The two cases involving other C-group chromosomes are both CML in acute phase This suggests, that the cytogenetic events occurring in the advanced phases of CML, which frequently leads to trisomy C [10], are of a different character than those leading to the trisomy C often described in other myeloid disorders including acute myeloblastic

leukemia GAHRTON *et al* [5] have shown, however, that trisomy 8 also occurs in blastic crisis of CML

Acknowledgments The laboratory work was supported by grants from Ferdn Hindsgaul s Foundation, P Carl Petersen s Foundation, Anders Hasselbalch's Fund against Leukemia and Danish Medical Research Council

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Book Review · Buchbesprechung · Livre nouveau

HORST STOBBE *Untersuchungen von Blut und Knochenmark;* 2 Aufl VEB Verlag Volk und Gesundheit, Berlin 1974 319 pp, 70 Abb, 25 Tab, 6 Farbtaf, M 20 40

Gegenuber der ersten Auflage des in der deutschsprachigen Literatur einmaligen Kompendiums der technischen Hamatologie bringt diese zweite Auflage erwartungsgemass zahlreiche Erweiterungen und Verbesserungen Dies wird besonders in den ersten Kapiteln deutlich Eine Abhandlung, die fruher auf Seite *100* stand, findet sich jetzt auf Seite 150 Ausführlich werden Methoden der Zellisolierung und -gewinnung besprochen, grossen Raum nehmen die zytochemischen Verfahren die durch instruktive Schemen erlautert werden ein Dabei fallt die Verwendung einer auf 0–4 reduzierten Aktivitatsstufeneinteilung zur Ermittlung des AI P Indexes auf In mühevoller Kleinarbeit sind überall dort Korrekturen angebracht worden, wo es der veranderte Stand der wissenschaftlichen Erkenntnisse erforderte Aus Gründen separater Veroffentlichung bleibt das Thema «Coagulation» weiterhin ausgeschlossen, jedoch wird jetzt die Thrombozytenfunktionsprüfung beschrieben Vollig neu ist der Abschnitt uber die Qualitatskontrolle im hamatologischen Labor, der alles Wissenswerte enthalt und von Spezialisten bearbeitet wurde

Insgesamt gesehen ist durch die besondere didaktische Befahigung des Autors auch mit dieser zweiten Auflage ein Werk von hohem Aussagewert für jeden der sich in Forschung Klinik und Praxis mit der Hamatologie beschaftigt, geschaffen worden, so dass dem Buch eine schnelle und weite Verbreitung nur gewünscht werden kann

F HECKNER, *Einbeck*

Varia

16th International Congress of Hematology

Kyoto Japan September 5–11, 1976

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Symposium held at the 15th Congress of the
International Society of Hematology in Jerusalem, September 1974

Nucleic Acids in the Pathogenesis of Leukemia

Guest Editor
Elio E. POLLI, Milano



S. Karger · Basel · München · Paris · London · New York · Sydney

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Nucleic Acids in the Pathogenesis of Human Leukemia

Introductory Remarks

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During these last decades basic science has brought some advances to the etiology of experimental and human leukemia As far as human leukemia is concerned, the following two hypotheses are now being considered Either the leukemic cell is considered a tumor cell without any possibility of regression to a normal condition, or the disease depends on the effect of some endogenous factors (such as CSF and chalones) on cellular proliferation and differentiation

The first hypothesis attracted many scientists since Virchow's time After the finding that new genetic information can be introduced into the cell by viral DNA or RNA, evidence has accumulated indicating that viruses can account for permanent transformation of the white cell to a malignant state However, some hematologists consider leukemia to be an array of different disorders, therefore the demonstration of viral involvement in the etiology of certain types of leukemia will not solve the problem of leukemia's etiology

In some cases of leukemia the virus is considered a necessary contributor, but in other cases the disease is dependent on the exposure to radiation or, alternatively, on other factors such as genetic disposition and immunological deficiency The possibility that two or more causes may be necessary is suggested by the lack of contagiousness of leukemia (perhaps except for clusters)

A clinician with an open mind may therefore accept a multiple etiology for leukemic disease, however, he is also convinced of a unique pathogenesis At the present state of our knowledge EB virus has been closely related with Burkitt's lymphoma, the same virus, or one closely related, causes infectious mononucleosis which was considered by Da-

FLV) to RNA of avian oncornaviruses. Probe DNA of the reaction resulting from leukemic reverse transcriptase and 70S RNA of MLV was shown to be hydrogen-bonded to viral RNA and also to anneal with homologous viral RNA. Labelled DNA by the DNA polymerase and its associated RNA from leucocyte fractions of patients with AML were hybridized on phosphocellulose filters with RNA of MLV, FLV, and ALV. About 10% of probe DNA hybridized with MLV, but showed no detectable homology with FLV and ALV. Furthermore, immunological studies suggest a relationship between DNA polymerases purified from human leukemic cell and reverse transcriptase of primate and mouse leukemia oncornaviruses. These results of molecular hybridization and of the immunological relationship support the findings from SPIEGELMAN's group which was able to detect MLV-specific sequences in leukemic cells.

All of you are aware that some problems are still unresolved, such as the presence of reserve transcriptase activity in only about 30% of patients with acute leukemia and the unknown relationship between the new DNA sequences and the disease. Comparisons between leukemic cells and immature normal white cells have not yet been made and TEMIN's researches have emphasized the presence of reverse transcriptase in uninfected chick embryo cells. However, as far as human leukemia is concerned, it seems possible to take an oversimplified view of leukemogenesis by both DNA and RNA viruses since after the initial reverse transcription of oncorna virus RNA to DNA, integration and subsequent expression of viral information in oncogenesis may be very similar.

These conclusions raise the question of a major role of nucleic acids in the pathogenesis of human leukemia.

After CHARGAFF, in the second half of the forties, first evidenced nucleic acid heterogeneity in different species, many investigators have tried to determine whether the neoplastic deviation could be correlated with abnormalities in the synthesis or physicochemical condition of nucleic acids. However, in these last years this hypothesis seems to be supported by some experimental evidence. Some of this evidence has been summarized above and will be discussed in detail in the present workshop. Other interesting data are coming from physicochemical studies on RNA and DNA isolated from both normal and leukemic cells. Recently, messenger RNA of normal, PHA and leukemic lymphocytes have been thoroughly studied in some laboratories. TORELLI will discuss recent development in this field in our workshop. Of interest seems to be the dis-

covery of a high percentage of repeated sequences (polyA) in messenger RNA of leukemic cells, an unusual condition of nuclear RNA, and most impressive are the findings showing an accumulation of double-stranded RNA in the nucleus of leukemic lymphocytes As is known, double-stranded RNA increases in the cell after viral infection and it is synthesized on DNA nucleotide sequences having high repetitive frequencies

Physicochemical studies on normal and leukemic DNA have shown that it contains families of nucleotide sequences with different repetitive frequencies The most highly repeated sequences are satellite DNAs CORNEO in our laboratory showed the presence of four satellite DNAs in normal and leukemic cells The biological role of satellite DNAs randomly distributed in all chromosomes is not definitely known However, CORNEO demonstrated that at least two human satellite DNAs are located near the centromeres of some human chromosomes Therefore, an attractive hypothesis concerning the biological role of satellite DNAs considers that they might be related to the organization of chromosomes and cell replication In acute leukemia, in which the replication processes of the cells appear to be modified, the study of satellite DNA might be of particular interest and CORNEO will discuss the work in progress

Other repeated nucleotide sequences having lower reassociation velocity than the very fast-renaturing satellite DNA have also been isolated and studies on the origin and function of such intermediate DNAs are being carried out in a number of laboratories As we will discuss later, we are interested in the problem of viral information possibly being integrated in these repeated sequences

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Biochemical Approach to Bovine Leukemia

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Key Words Bovine leukemia Leukemia virus Molecular hybridization Virus production

Abstract Many bovine leukemic lymphocytes produce virus particles when kept in survival cultures in Eagle s Minimum Essential Medium supplemented with 20% of inactivated fetal calf serum Virus particles equilibrate at a density of 1 16 g/ml in sucrose gradients and at a density of 1 12 g/ml in metrizamide gradients Simultaneous detection tests show that a high molecular weight RNA reverse transcriptase complex exists in these viruses Hybridizations between total RNA from bovine leukemic lymphocytes and C DNA prepared in various known RNA oncogenic viruses show that the virus associated with bovine leukosis is unrelated to RLV SSV 1 MSV Ki FSV Ga and FLV Ri

Enzootic bovine leukosis was diagnosed in Belgian cattle in 1966 [2, 4] This tumoral disease is not uniformly spread in the country [5, 6] Extensive field and experimental studies have been performed Some of them deal with natural and experimental transmission of the disease [7–9] The conclusion of this research is that vertical as well as horizontal transmissions are responsible for the spreading of the disease These observations together with those of other research groups [1, 10–12]

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Fig. 1. Thin section of a cultured lymphocyte from a leukemic cow. N = Nucleus; M = mitochondrion; V = viruses. × 30 000

point to a viral etiology of bovine leukosis. The putative agent of the disease is called bovine leukemia virus.[4]

In the recent years, evidence has accumulated that human neoplasms might also have a viral etiology [15]. The involvement of RNA tumor

[4] Abbreviations used: BLV = bovine leukemia virus; AMV = avian myeloblastosis virus; RLV = Rauscher leukemia virus; SSV 1 = simian sarcoma virus 1; MSV Ki = murine sarcoma virus Kirsten strain; FSV Ga = feline sarcoma virus Gardner strain; FLV Ri = feline leukemia virus, Rickard strain; MPMV = Mason Pfizer monkey virus; DTE = dithioerythritol; NP-40 = nonionic detergent P-40 from Shell Co.; ^{3}H TTP = thymidine triphosphate sodium salt ^{3}H labeled; dATP = deoxyadenosine triphosphate sodium salt; dGTP = deoxyguanosine triphosphate sodium salt; dCTP = deoxycytidine triphosphate sodium salt; dT_{12-18} = oligodeoxythymidylic acid 12–18 residues; C DNA = DNA synthesized as the complement of an RNA template; EDTA = sodium salt of ethylene diamine tetra acetic acid; TCA = trichloroacetic acid



Fig 2 Same culture as in figure 1 The arrows indicate virus particles where hexagonal contours are best illustrated × 60 000

viruses in human tumors is better documented by electron microscopy together with a biochemical approach involving simultaneous detection tests and molecular hybridization of nucleic acid molecules We used both types of approaches in the experiments reported here

Methods

Cell cultures and preparation of samples for electron microscopy as described by MAMMERICKX and DEKEGEL [9]

Virus purification The culture was centrifuged at 800 *g* for 20 min Cells were washed in TNE (0 01 M Tris HCl pH 8 3 0 15 M NaCl 0 001 M EDTA) and again pelleted at 800 *g* They were then resuspended in 4 vol of 0 01 M EDTA at 0 °C, homogenized by 4–6 strokes in a Dounce type B homogenizer and submitted to 30 sec of ultrasonic vibration (8-ml sample maximum power of a Mullard vibrator) The homogenate was then successively spun at 4 000 *g* for 10 min and 10 000 *g* for 10 min The supernatant was then layered onto a column of 20% sucrose in TNE and centrifuged for 1 h at 98 000 *g* and 4 C.

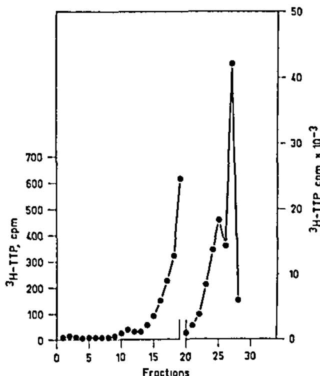


Fig 3 Sedimentation velocity profile of the C-DNA synthesized in a 30 min reaction using the 98,000 *g* pellet as a source of high molecular weight RNA and reverse transcriptase Fractions 8–14 correspond to the 50–70S region The gradient is a 10–20% sucrose in TNE Leukemic white blood cells were cultured for 16 h

Simultaneous detection tests were run essentially as described by SCHLOM and SPIEGELMAN [14]

^{3}H C-DNA synthesis The reaction mixture was as follows (final volume = 2 ml) Tris HCl, pH 8 3 0 05 M, DTE 0 0005 M, NaCl 0 04 M, $MnCl_2$ 0 001 M, dATP, dCTP, dGTP 0 02 mM each, ^{3}H TTP 3 mCi (SA = 50 mCi/μM), viral protein 300 μg/ml, NP-40 0 025%, Act D 100 μg/ml Synthesis was run for 90 min at 37 °C and the product purified as described by SPIEGELMAN *et al* [16]

Hybridizations and Cs_2SO_4 gradient analysis as outlined by SPIEGELMAN *et al* [16]

Results

Figures 1 and 2 illustrate the morphology of virus particles appearing in cultures In figure 1 virus particles are aligned along the cytoplasmic membrane of a lymphocyte In figure 2 the geometrical shape of the parti-

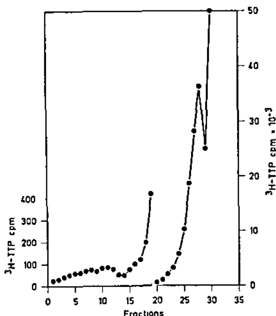


Fig 4 As figure 3 but cells were kept in culture for 48 h

cles is best illustrated by the particles indicated with an arrow The contours of both the particle and the nucleoid look hexagonal The outer membrane seems to be double

Figures 3 and 4 show the outcome of simultaneous detection tests performed on the 98,000 *g* pellet Fractions 8–14 represent the high molecular weight RNA region A small but significant number of counts sediments in that region Panels (fig 3, 4) were obtained, respectively, from cells cultured for 16 and 48 h

To further characterize the viral fraction and its components, we performed the experiments illustrated on figures 5 and 6 The 6 panels of figure 5 represent the sedimentation profile of the nucleic acid material recovered from the material banding at the indicated density regions of a sucrose gradient This material was used in a reverse transcriptase reaction and nucleic acids were extracted and analyzed by sedimentation velocity There is a significant number of TCA-precipitable counts in the

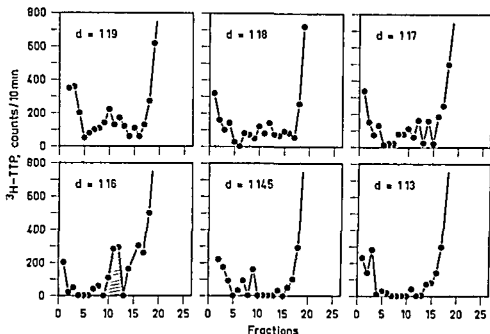


Fig 5 Sedimentation velocity profiles of the C DNA synthesized in a 30 min reverse transcriptase reaction using the cellular material equilibrating at the indicated densities (g/ml) as a source of template and enzyme The equilibrium density centrifugation was run in a 20-70% sucrose gradient in TNE The sedimentation velocity gradient is a 5-20% sucrose in TNE

high molecular weight RNA region of two gradients, one corresponding to the material of a density of 1 16 g/ml and one corresponding to the material of a density of 1 19 g/ml We therefore conclude that viral material exists in structures banding at these two densities in sucrose gradients

In a parallel experiment, metrizamide was used as a centrifugation medium instead of sucrose AMV used as a marker banded at ϱ = 1 12 g/ml and nucleoproteins equilibrated at lower densities From the data shown on figure 6, we conclude that viral material was present in the density region of 1 12 g/ml and probably also in the region of 1 108 g/ml (*perhaps as disrupted viruses*)

Another series of experiments aimed at clarifying a possible relatedness between the bovine virus and other known RNA oncogenic viruses such as RLV, SSV-1, MSV-Ki, FLV-Ri, and FSV-Ga The C-DNA prepared in each one of these viruses was hybridized to total RNA from cultured bovine leukemic lymphocytes No hybrid structure could be detect-

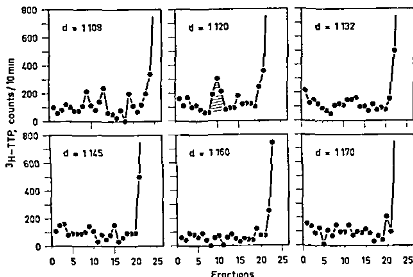


Fig 6 Same experimental scheme as in figure 5, except that the equilibrium density gradient is a 15–50% metrizamide in TNE

ed in these experiments We are thus lead to the conclusion that the bovine agent, BLV, is unrelated to the viruses tested

Discussion

The simultaneous detection tests performed allowed us to identify viral material before and after equilibrium density gradient centrifugations However, the total number of counts sedimenting in the high molecular weight RNA region of sucrose gradients remained persistently small Two possible explanations can be considered (1) RNA of our BLV is largely degraded and RNA DNA hybrids sediment slower than 50–70S Indeed, if top fractions of velocity gradients are analyzed on Cs_2SO_4 gradients, a significant proportion of counts equilibrates in the RNA region (2) Reverse transcriptase of BLV produced in our conditions is poorly active This possibility is currently being checked

Our conclusion that BLV is unrelated to RLV, SSV-1, MSV-Ki, FLV-Ri, FSV-Ga, is based upon hybridization experiments between total

cellular RNA of bovine leukemic lymphocytes and C DNA prepared in the above specified viruses As 300 μg of RNA are used per hybridization and no hybrid structures are detected we conclude, considering that we are able to detect easily 100 cpm in a gradient that there is less than 10 ng of sequences, possibly related to the viruses tested More experiments with purified BLV are required before decisive conclusions are reached but we can already say that the relatedness between BLV and the viruses used if it exists, must be extremely small This observation is in agreement with the immunological data obtained by others and showing that BLV might represent a new group of mammalian oncorna viruses [3, 13]

Acknowledgements We warmly thank Dr J BEARD (Life Sciences St Petersburg Fla) who supplied us with AMV and Dr J GRUBER (NCI Office of Program Resources) for a generous gift of SSV 1) This work was supported by the Fonds cancérologique de la caisse générale d Epargne et de Retraite

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Acta haemat 54 210–220 (1975)

Human Leukemic Intermediate DNA Components

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Key Words CsCl centrifugation DNA renaturation Intermediate DNA Leukemic leucocytes MAK column chromatography Repeated DNA in human genome

Abstract The DNA extracted from human leukemic leucocytes has been fractionated on a methylated albumin kieselguhr (MAK) column The different fractions obtained have been reannealed to a Cot value of 20 mol×sec/l to study the distribution of the intermediate DNA on the MAK column Intermediate DNA contains two components one (CsCl density after reannealing 1 703 g/ml) obtained by reannealing high molecular weight DNA the other (CsCl density after reannealing 1 707 g/ml) obtained only by reannealing sonicated low molecular weight DNA High molecular weight intermediate DNA (1 703 component) is eluted early from the MAK column in the fractions corresponding to the main DNA peak while low molecular weight intermediate DNA (1 707 component) is more widespread on the MAK column but appears to be enriched in the fractions eluted later The possibility is discussed that the latter component is interspersed in that part of the genome which is apparently more homogeneous in density in an analytical CsCl gradient and is absent on the skewed more heterogeneous, heavy side of the main DNA in CsCl

Eukaryote DNA contains a small amount of very highly repeated nucleotide sequences, some other less repeated nucleotide sequences, called intermediate DNA and unique not repeated nucleotide sequences [1] This has been established by fractionation of reassociated DNA on hydroxyapatite columns and by DNA optical reassociation curves Some satellite bands obtained from the native nuclear DNA of several species by density gradient centrifugation have been shown to correspond to the very highly repeated nucleotide sequences demonstrated by hydroxyapatite column chromatography of reassociated DNA Less highly repeated

nucleotide sequences have been identified by CsCl density gradient centrifugation of DNA reannealed to intermediate values of Cot in human [7, 10, 17] mouse [2] and *Drosophila* [11–13] genomes We describe in the present paper an attempt to study the elution of human intermediate DNA on a methylated albumin kieselguhr (MAK) column

Materials and Methods

Fractionation of DNA on MAK columns The DNA was extracted from human leukemic leucocytes according to a modification of Marmur's [15] method as previously reported [3] The DNA obtained had a molecular weight of about 15 million daltons in the double-stranded form as determined by band sedimentation [18] MAK column chromatography was carried out as previously described [8] The DNA was dialyzed against 0.5 M NaCl-0 05 M sodium phosphate buffer (pH 6.8) and then loaded on the column ready to be used at a concentration of 20 μg/ml A column of a size of 40×4 cm was loaded with 7–10 mg DNA. The continuous gradient produced by a gradient making device had a molarity of 0.5–0 8 M NaCl 0 05 M sodium phosphate buffer and a total volume of 800 ml The flow rate was 20 ml/h and the fractions obtained had volumes of 10–15 ml The salt concentration of the eluates was checked by refractive index measurements. The fractions were collected by an LKB (Stockholm Sweden) fraction collector The recovery of the DNA loaded was higher than 95% as judged from the ultraviolet absorption Smaller columns 13×1.5 cm were used to fractionate further some DNA fractions eluted from the first column These smaller columns were loaded with 2–2.5 mg of DNA, which was eluted at a flow rate of 20 ml/h with a continuous gradient of 0.5–0 6 M NaCl 0 05 M phosphate buffer (pH 6.8) The fractions collected had volumes of 5 ml each.

Reannealing of DNA Sonication of native DNA in 1.5–2 ml of 0 1×SSC was carried out by a MSE sonicator at 10 A and 20 000 cps for 2 min at intervals of 30 sec The DNA was reduced in this way to a molecular weight of 300 000 daltons as determined by band sedimentation [18] The DNA was denatured by heating for 10 min at 100 °C in 0 1×SSC (SSC = 0 15 M NaCl 0 015 M sodium citrate) and was then reannealed by heating at 65 °C in 2×SSC at a Cot value of 20 mol×sec/l [1]

Density gradient centrifugation 1 5 μg DNA were centrifuged in analytical equilibrium CsCl gradients, as described [5] using *Streptomyces mediterraneus* DNA (density 1 730 g/ml) as reference All DNA densities are referred to the density of *Escherichia coli* DNA, taken to be 1 710 g/ml

Absorbance temperature curves of DNA were carried out in 1×SSC as described previously [5]

Results

In previous papers [7, 10] we have shown that human total DNA when reannealed to a Cot value of 20 mol×sec/l after heat denaturation

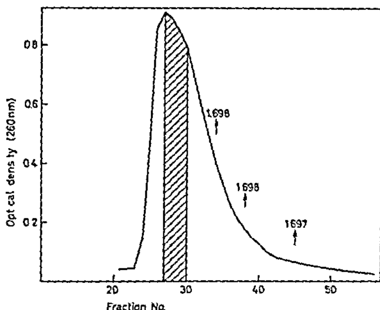
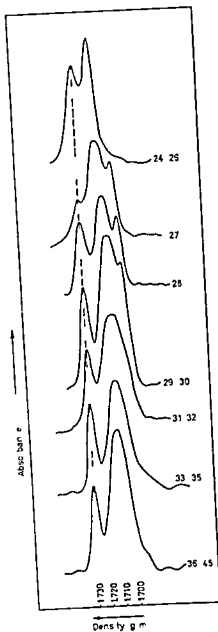


Fig 1 Fractionation of human leukemic leucocyte DNA on a MAK column The hatched region corresponds to the DNA which has been further fractioned on a subsequent MAK column (see fig 4) The native CsCl density of some fractions is indicated along the profile

shows in analytical neutral CsCl at equilibrium some DNA which has moved back towards the native density This rapidly renaturing DNA forms a band with a density of 1 703 g/ml in CsCl when high molecular weight DNA is reannealed If the DNA is reduced to low molecular weight by sonication before annealing, an additional band of reassociated DNA with a density of 1 707 g/ml can be shown in the CsCl gradient These two bands of reassociated DNA account for 7 and 10% of the total DNA, respectively, and here will be called 1 703 and 1 707 DNA components We describe here an attempt to study the distribution of these two

Fig 2 Microdensitometer tracings of pools of fractions of DNA obtained from the MAK column experiment reported in figure 1 reannealed without previous sonication centrifuged to equilibrium in CsCl in the analytical ultracentrifuge The fractions were pooled so that the DNA is subdivided into 7 portions eluting progressively from the MAK column and containing approximately equal amounts of DNA The numbering of the fractions corresponds to that of figure 1 The peak on the left in each tracing in this figure and figures 3 and 5 corresponds to the standard *Streptomyces mediterraneus* DNA (density 1 730 g/ml)


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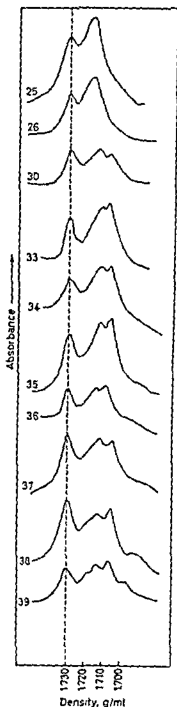


Fig 3. Microdensitometer tracings of different fractions of DNA obtained from the MAK column experiment reported in figure 1, reannealed after previous sonication, centrifuged in analytical CsCl The numbering of the fractions corresponds to that of figure 1

components of reassociated DNA in the different fractions of human DNA obtained by elution from a MAK column

Figure 1 shows the fractionation of the DNA obtained from human leukemic leucocytes on a MAK column Equal amounts of each fraction were pooled so as to obtain pools of fractions, each containing similar amounts of DNA These were then centrifuged to equilibrium in analytical CsCl after heat denaturation followed by renaturation at a Cot value of 20 mol×sec/l at 65 °C in 2×SSC without previous sonication, as shown in figure 2 The CsCl patterns of fractions 27–30 of figure 2 show a band, which judging from the decrease in buoyant density after annealing appears to correspond to the 1 703 component, beside the main band of denatured DNA

Small aliquots of single DNA fractions obtained from the MAK column experiment reported in figure 1 were also centrifuged to equilibrium in analytical CsCl, after heat denaturation and reannealing in the conditions indicated above with previous sonication (fig 3) The renatured DNA present in these conditions in the fractions 27–30 corresponds to the 1 703 component already demonstrated in the fractions of figure 2 However, the CsCl patterns of the fractions reannealed after sonication show that also in the fractions eluted late (33–45) a peak of renatured DNA appears in these conditions beside the denatured DNA peak This DNA, which corresponds to the 1 707 component, is enriched in the fractions eluted late from the column and is absent in the fractions eluted early

We have made an attempt to purify at least the 1 703 component in native conditions We have pooled the fractions eluted from the MAK column corresponding to the hatched area in figure 1, and we have eluted them from a smaller MAK column with a flat salt gradient The result obtained is reported in figure 4, where the hatched area corresponds to the elution of the 1 703 component as judged from the centrifugation in CsCl of different fractions submitted to renaturation without previous sonication (right side of fig 5) We obtained an enrichment of up to 40% of the 1 703 component in some fractions It is remarkable that the 1 703 component has a restricted localization on the MAK column Comparing the *intermediate DNA contained in the several fractions of figure 5*, it can be seen that only in fractions eluted late (fraction 63 of fig 5) a band of 1 707 component is present, in addition to denatured DNA and to the 1 703 component. This latter component, on the other hand, is absent in fractions eluted later (fraction 67)

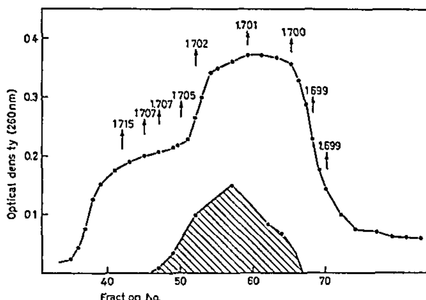


Fig 4 Fractionation of a pool of fractions 27-30 obtained from the hatched part of the elution profile of DNA in the MAK column experiment reported in figure 1 on a subsequent MAK column The hatched peak shown here corresponds to the elution pattern of 1 703 component The relative amount of 1 703 component has been evaluated by estimating the area under the reassociated DNA peak in each fraction reannealed without previous sonication and centrifuged in CsCl as reported in figure 5 (right side) The native CsCl density of some fractions is indicated along the profile

From the native densities of the fractions containing the higher percentage of intermediate DNA reported in figures 1 and 4 we presume that the native densities of intermediate DNA are approximately 1 701–1 702 g/ml and 1 698–1 699 g/ml, respectively, for the 1 703 and 1 707 components *It is remarkable that also the 1 707 component which renatures* only after previous disruption of DNA by sonication is not distributed at random along the genome, but appears to be enriched in the fractions eluted late from the MAK column Even if the MAK column is not an ideal fractionation procedure at least the fractions eluting early, corresponding to the skewed high density side of the DNA main band in CsCl [3], do not appear to contain 1 707 component

We have also carried out a melting curve of some of the fractions obtained from the MAK column experiment reported in figure 1 The fractions eluted late containing approximately 50% of 1 707 component, dis

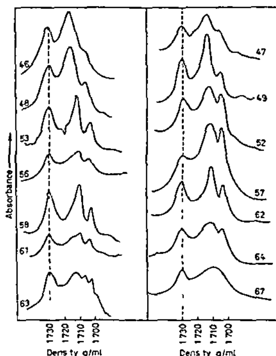


Fig 5 Microdensitometer tracings of some fractions of DNA obtained from the MAK column experiment reported in figure 4, reannealed with (left) and without (right) previous sonication, centrifuged in analytical CsCl The numbering of the fractions corresponds to that of figure 4

play a melting curve after reannealing similar to that obtained in the same conditions with the isolated reassociated 1 707 component [10]

Discussion

Some considerations can be made on the basis of the experiments here described Human DNA contains intermediate DNA demonstrated by CsCl centrifugation of reassociated DNA and corresponding to some of the intermediate DNA isolated by hydroxyapatite fractionation of reassociated DNA [17] The 1 703 component forming long stretches along the

genome has a restricted localization on the elution profile from the MAK column while the 1 707 component eluted later on the MAK column, is more finely but not evenly distributed

Intermediate DNA differs in some respects from the very highly repeated or simple-sequence satellite DNAs In fact it cannot be separated from the main band as a satellite band in native conditions [5, 6], does not renature as fast as satellite DNAs, and does not separate into the two complementary strands in alkaline CsCl as most satellites do [4–6] For several reasons it can be excluded that the intermediate DNA studied here corresponds to the previously known human satellite DNAs

These account for too small a percentage of the total DNA to be visible in the CsCl gradients in the conditions used here [4–6] When the analytical cell is filled with 20 μg of reassociated total human DNA, a very faint band of DNA, having a density of 1 698 g/ml, is also evident in the CsCl profile of human reassociated DNA [7, 10] This band might correspond to satellite II [7, 10] Furthermore when the very light fractions obtained from the preparative CsCl centrifugation of reassociated DNA are pooled and then concentrated by slow evaporation and centrifuged in CsCl in the analytical ultracentrifuge, another band having a density of 1 688 g/ml is shown, probably corresponding to satellite DNA I [7, 10] Intemediate DNA when centrifuged in alkaline CsCl in the analytical ultracentrifuge does not separate into the two complementary strands [10]

The two components of intermediate DNA here described cannot correspond to 'folding-back' of single stranded DNA containing a sequence inversion [2] This 'fold-back' DNA binds to hydroxyapatite at a very low Cot value and is not due to a bimolecular renaturation process It does not band sharply in CsCl [2], while on the other hand the intermediate DNA, studied here, is evident in the CsCl gradient only after reannealing at a Cot value higher than 10 and forms sharp bands in CsCl Furthermore it has been shown that these DNA components are eluted from a hydroxyapatite column only at intermediate values of Cot [17] Therefore we conclude that the DNA studied here is different from simple-sequence satellite DNAs and does not correspond to 'fold-back' DNA Another main distinguishing characteristic between satellite DNAs and intermediate DNA is that the latter is very likely transcribed *in vivo*, while satellite DNAs are not transcribed [MELLI *et al*, in press] Intermediate DNA forms stretches of high molecular weight along the genome or is more finely distributed, although even in this case it appears to be enriched in some portions of the genome Using completely different techniques other

authors reached the same conclusion that interspersion of repeated sequences is different in different parts of the eukaryote genome [9, 14, 16]

Here we suggest on the basis of the present experiments that the highly interspersed intermediate DNA is enriched in the portion of DNA eluting late from a MAK column It is interesting to note that this DNA corresponds to the portion of DNA which is apparently more homogeneous in density in an analytical CsCl gradient [5]

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Hybridization of Mouse Leukemia Virus c-DNA to Mouse Repeated DNA Sequences

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Key Words Leukemia virus Satellite DNA Intermediate DNA Integration of provirus Mouse leukemia

Abstract Experiments of hybridization between mouse leukemia virus synthetic ^3H DNA probe and mouse main band and satellite DNAs indicate that there is not a higher concentration of viral sequences in the satellite DNA. On the contrary viral sequences appear to be enriched in the fast renaturing intermediate main band DNA

Some preliminary experiments have been carried out in order to establish whether DNA copies (or provirus) of oncornavirus genomes are integrated in a mouse-transformed cell line preferentially in the satellite or other repeated sequences

Materials and Methods

The transformed mouse cell line 1798 (which produces both MSV(H), mouse sarcoma virus Harvey strain, and MLV) obtained by infection of a normal mouse cell line (3T6 clone 91) with H MSV (MLV) was used Mouse 3T6 (clone 91) cells were used as normal cells [8] Both cell lines were provided by M GREEN

The cells were grown as monolayers in Dulbecco s modified Eagle s medium containing 10% fetal calf serum Viral DNA probe was prepared by the endogenous RNA-directed DNA polymerase reaction of detergent (nonidet P40) disrupted H MSV (MLV) utilizing ^3H TTP as the labeled substrate in the presence of 200 mg/l of actinomycin D [6] The viral c DNA used was the single stranded DNA obtained after self annealing at a Cot value of 1 mol×sec×liter^{-1} by hydroxyapatite

Cell DNA was extracted by MARMUR s method [7] Mouse satellite DNA was isolated from main band DNA by preparative silver-cesium sulfate centrifugation of

native DNA [2] or by cesium chloride centrifugation of denatured and reannealed DNA to low Cot value [12] Mouse fast renaturing main band DNA [1] was isolated by preparative Ag^+ Cs_2SO_4 centrifugation of reassociated DNA to intermediate Cot value [5]

Viral DNA probe was hybridized to isolated mouse satellite and main band DNA First the DNA was degraded to low molecular weight (approximately 200 000 daltons single stranded) by boiling in 0 3 N NaOH for 20 min The hybridization was carried out in solution at 60 °C in 0 48 M phosphate buffer (pH 6 8) up to a Cot of 10 000 [9 11] The amount of radioactive viral c DNA hybridized was evaluated by batch elution at 60 °C of single stranded and double-stranded DNA from hydroxyapatite [10]

Melting profile of the hybrid obtained after reaching a Cot of 10 000 was carried out by separating single-stranded from double-stranded DNA radioactivity, in samples obtained at different temperatures on hydroxyapatite with 0 12 M phosphate buffer

Further preliminary experiments were carried out using DNA/DNA filter hybridization technique [3] Cell DNA fractions obtained by preparative centrifugation in Ag^+ Cs_2SO_4 were loaded on filters in alkaline solution containing 2 M thiosulfate as silver complexing agent at very low DNA concentration after alkaline denaturation at pH 13 Hybridization was carried out at 60 °C in 4×SSC for 96 h in Denhardt solution (0 01% ficoll 0 02% polyvinylpyrrolidone, 0 02% bovine albumin fraction V) after preincubation of the filters At the end of the reaction filters were washed in 2×SSC.

Results

The preliminary results obtained in the experiments of hybridization between the viral DNA probe and the isolated main band and satellite DNAs in solution indicate that there is not a higher concentration of viral sequences in the satellite DNA The hybridization between the viral probe and the satellite DNA is not due to highly reiterated sequences present in the viral genome, according to the Cot values obtained

In figure 1 the melting curves obtained by thermal elution of hybrids between the viral probe and satellite and main band DNA from hydroxyapatite are shown There is no significative difference between main band and satellite DNA, while there is an evident difference in thermal stability in the hybrids obtained with transformed and normal DNA The higher thermal stability of the hybrid obtained with transformed DNA is likely due to a higher degree of molecular homology with the viral probe This is in agreement with previous observations [11]

In figure 2 the pattern of mouse total DNA (molecular weight 1,500,000 daltons single stranded) after denaturation and renaturation to

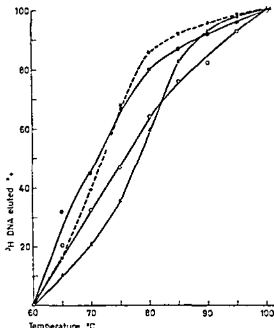


Fig 1 Melting curves of the hybrids between H MSV (MLV) ^{3}H DNA probe and isolated main band and satellite DNAs of mouse transformed (cell line 1798) and normal cells The percent of ^{3}H DNA eluted as single strand from hydroxyapatite with 0 12 M phosphate buffer is plotted against temperature ▲ = 1798 main band ○ = 1798 satellite • = normal main band ● = normal satellite

a Cot of 20 at equilibrium in the analytical ultracentrifuge is shown. In the upper tracing the DNA has been centrifuged in neutral CsCl, in the lower tracing the DNA has been centrifuged in Ag^+ Cs_2SO_4 The peak on the right corresponds to the satellite DNA, the peak on the left corresponds to the main band DNA, and the shoulder on the right side of the main band corresponds to fast renaturing main band DNA (FRMB)

In figure 3 total DNA obtained from mouse transformed cells, reassociated to a Cot value of 20 after heat denaturation, has been fractionated on a preparative Ag^+-Cs_2SO_4 gradient The three peaks from the left correspond to main band, fast renaturing main band and satellite DNAs The radioactivity bound to different fractions after filter hybridization with the viral DNA probe is also reported As shown particularly by the specific

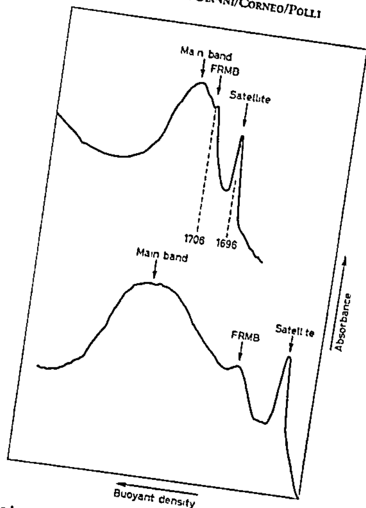


Fig. 2. Microdensitometer tracings of mouse cell DNA, reassociated to a Cot value of 20 after denaturation, centrifuged to equilibrium in the analytical ultracentrifuge, in CsCl (upper tracing) and in Ag^{+}-Cs_2SO_4 (lower tracing).

activity of the fractions, viral sequences appear to be enriched in the fast renaturing intermediate main band DNA (fig. 4).

Recent work which came to our attention after this communication had been presented, indicates that avian leukemia virus sequences hybridize to intermediate DNA of normal and leukemic avian tissue culture cells (4).

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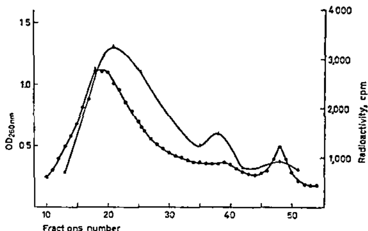


Fig 3 DNA/DNA hybridization between mouse transformed cell (line 1798) DNA, fractionated in a preparative Ag^{+}-Cs_2SO_4 gradient at equilibrium, and H MSV (MLV) ^{3}H DNA probe ● = Optical density, △ = radioactivity

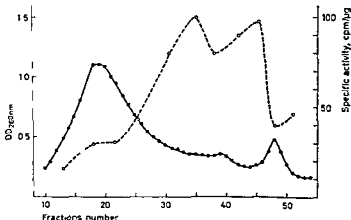


Fig 4 Fractionation of mouse transformed cell (line 1798) DNA in a preparative Ag^{+} Cs_2SO_4 gradient at equilibrium. The dotted line corresponds to the specific activity of different fractions after hybridization ● = Optical density

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Genome Complexity and *in vivo* Transcription in Human Leukemic Leukocytes

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Key Words Satellite DNA Sequence arrangements Cellular RNA Chromatin transcription Leukemic leukocytes

Abstract Human DNA contains 35% repetitive and 65% nonrepetitive sequences Eight satellite DNAs have now been identified in human DNA The highly repetitive non satellite sequences are arranged in blocks 1 100 nucleotides long and interspersed with intermediate repetitive and nonrepetitive sequences greater than 2,500 nucleotides long Approximately 2.5–3 9% of the nonrepetitive sequences are represented in total cellular RNA of normal and leukemic leukocytes

The potential information content of the human genome is tremendous For example, if all the DNA coded for proteins there would be enough information to code for approximately 7 million proteins The question of how much of the DNA is transcribed *in vivo* and how much of it is transcriptionally inactive is germain to our understanding of the regulation of gene activity The genomes of most eukaryotes contain DNA sequences present once per haploid genome (single-copy or unique DNA) as well as multiple-copy or repetitive sequences For experimental purposes, it is convenient to divide the human genome into repetitive and nonrepetitive sequences [13]

Before examination of the transcriptional activity of the different kinetic classes of DNA, we would like to say a few words about satellite DNAs Satellite DNAs are operationally defined as fractions which band in density gradients at positions removed from the main band At least 8 well-defined satellites have been isolated from the human genome and in

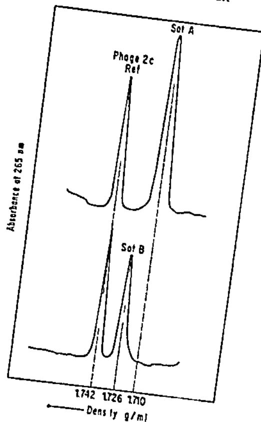


Fig 1 Buoyant densities of human satellite DNAs A and B Satellite DNAs A and B were isolated from the purified human nucleolar DNA using $Ag^+Cs_2SO_4$ density gradient centrifugation The molar ratio of Ag^+ to DNA phosphate was 0 5 and the DNA concentration was 200 µg/ml Both satellites A and B appear at the light side of main band DNA in the $Ag^+Cs_2SO_4$ gradient Each fraction is then purified through recycles of centrifugation in Cs_2SO_4 and CsCl In neutral CsCl density gradient centrifugation the purified satellite A shows a single sharp band at 1 710 g/ml, corresponding to 51% G+C content whereas satellite B bands heavier at 1 726 g/ml corresponding to 67% G+C content The sharpness of these bands is indicative of a high molecular weight DNA having a relatively homogeneous base composition Phage 2C DNA (ρ = 1 742 g/ml) is used as a density marker

some cases rather extensive studies of their sequence arrangements have been carried out For example, satellite A, which has a buoyant density of 1 710 g/ml (fig 1) gives a biphasic reassociation curve [3] The fast component reassociates 1,600 times more rapidly than the slow component Evidence demonstrating that the two kinetically different sequences are on the same DNA strand has been obtained from buoyant density analys-

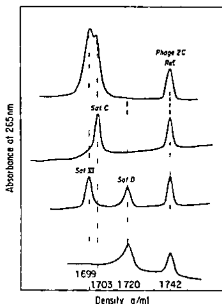


Fig 2 Buoyant densities of human satellite DNAs III, C, and D Satellites III, C, and D were isolated from the bulk human DNA using $Ag^{+}Cs_2SO_4$ density gradient centrifugation The molar ratio of Ag^{+} to DNA phosphate was 0 27 and the DNA concentration was $\lesssim 50\ \mu g/ml$ In neutral CsCl density gradient satellite C bands at 1 703 (2nd tracing), satellite D bands at 1 720 (4th tracing) with reference to phage 2C DNA (1 742 g/ml) The top tracing shows the density profile of the mixture of roughly equal amounts of satellites III and C Each peak corresponds to that of the purified fraction The third tracing shows the density profile of the mixture of satellites III and D Two peaks are well separated and each corresponds to the density of the purified fraction

es of partially reassociated satellite A Since the partially reassociated material did not split into two peaks, the different kinetic classes must be on the same DNA strand [3]

A summary of the physical properties of the human satellite DNAs is shown in table I Two human satellites III and C have similar buoyant densities (fig 2) and chromosome locations (C9 primarily) but satellite III contains a heptanucleotide repeating sequence [W SALSER, personal commun] and undergoes strand separation in alkaline CsCl, whereas satellite C gives a single band in alkaline CsCl A new satellite (satellite D) has recently been isolated It has a buoyant density of 1 720 g/ml (fig 2)

Taken together, these results suggest greater differences among classes of human lymphocytes in the repetitive transcripts than in the nonrepetitive transcripts. Leukemia appears to result from an impairment of the cell differentiation process. The site of blockage has not been defined, but within the context of the genetic control apparatus, it appears that impairment involves transcription of DNA sequences in malignant cells which are not transcribed in normally differentiated cells. Does this mean different messenger RNAs are synthesized in lymphoblasts than in the more differentiated lymphocytes such as CLL and NC-37? Or perhaps the differences are only in the nuclear restricted RNA populations? Two recent reports have dealt with the complexity of polyadenylated polysomal RNA (mRNA) of Hela cells. KLEIN *et al.* [11] have divided the mRNA into two classes. One class comprising 6% of the total appears to be transcribed entirely from repetitive DNA. The second class of mRNA (94% of the total mRNA) is entirely unique DNA transcripts. In a similar study, BISHOP *et al.* [1] found that approximately 1% of the single-copy DNA is transcribed into mRNA. This is enough to code for 35,000 proteins of average size. The unique-copy mRNA molecules are present in 3 distinct frequency classes, about 20% of the messenger RNA is transcribed from very few (17) sequences, about 30% of the mRNA is transcribed from 370 different sequences, and 50% of the mRNA is transcribed from 33,000 different sequences. With methodology now available, it is possible to determine the complexities of nuclear and messenger RNAs in normal and leukemic lymphocytes and this will soon be accomplished.

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Characteristics of Heterogeneous Nuclear RNA in Normal Small Lymphocytes and in Acute Leukemia Blast Cells

An Outline

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Key Words Lymphocytes Leukemic cell Heterogeneous nuclear RNA Repetitive sequences Poly(A) sequences Double stranded sequences

Abstract In this short review of the characteristics of heterogeneous nuclear RNA of normal small lymphocytes and of leukemic blast cells the author points out that the average life time of the molecules of this RNA class in both types of cell is much longer than that so far reported for rapidly proliferating cells In both types of cell the newly synthesized RNA includes rapidly hybridizing sequences There is evidence, although circumstantial, that in leukemic cells these sequences are markedly different from that of normal lymphocytes In both normal lymphocytes and leukemic blast cells the rapidly hybridizing RNA sequences are in part involved in secondary structure In fact, a significant fraction of the hnRNA appears in RNase resistant form with double stranded properties About one fifth of the hnRNA molecules of normal lymphocytes carries poly(A) segments, whereas this proportion is markedly higher in leukemic cells

A number of investigations over the past 10 years has been directed towards characterizing the heterogeneous nuclear RNA (hnRNA) of animal cells Among the most important discovered properties of this RNA class, we have to include the extreme metabolic instability, with an estimated life time of 5–15 min [2, 19], the presence of nucleotide sequences hybridizing to repeated sequences in cell DNA [4, 17], the presence of covalently linked sequences of polyadenylic acid of at least two different types short internal sequences, about 30 nucleotides long [12] and long, terminal, nontranscriptional sequences, about 150 nucleotides long [5, 6], and finally the presence of double-stranded regions [7, 9, 11, 14] Most of these investigations have been carried out in exponentially growing cells, such as the HeLa cells Of particular concern for the hematologist, how-

ever, are some types of non-growing cells, such as the small circulating lymphocytes and the circulating leukemic blast cells The characteristics of the hnRNA in these cells will be briefly outlined here

The small circulating lymphocytes and the circulating leukemic blast cells are obviously very different cell populations The former is mainly formed by homogeneous, long living cells with densely packed chromatin and scanty cytoplasm, the latter is formed by cells of various origin, usually of much larger size and cytoplasmic development, with large nu cleoli and very fine chromatin However, these two cell populations have some closely similar functional characters Both types of cells show a very low rate of cell proliferation, so that most of them are regarded as 'out of cycle' cells Furthermore, the average rate of RNA synthesis is very low in both types of cells [20] This is apparently due to the almost complete failure of processing of ribosomal precursor RNA in these cells In small lymphocytes [3, 21] as well as in leukemic blast cells [25, 26] *processing of the original 45S ribosomal precursor RNA molecule to the 32S intermediate and of this molecule to 28S occurs at a very low rate, and formation of 18S RNA is almost completely abortive, so that 'wastage' of this molecule occurs at a high degree [16, 26] Heterogeneous nuclear RNA* represents a conspicuous proportion of the total RNA synthesized in small lymphocytes [21, 22] as well as in many populations of leukemic blast cells [23] Some of the most peculiar features of this RNA in human small lymphocytes shall be shortly reviewed here

As far as the turnover rate is concerned, any evidence so far obtained indicates that the rate of degradation of the hnRNA molecules is very low Even after several hours of 'chase' with actinomycin D or with cold uridine, a considerable amount of radioactivity remains associated with molecules larger than 45S [21, 22] The average life time of these molecules seems to be in the order of some hours, at variance with that of hnRNA of rapidly proliferating cells [2, 19]

Some years ago we reported [21] that high molecular weight RNA synthesized in small lymphocytes showed a high efficiency of RNA DNA hybridization *in vitro* It has been shown, since then, that *in vitro* hybridization of DNA and RNA from animal cells under the usual conditions permits mainly the hybridization of repetitive gene fractions [10] We can thus conclude that the RNA synthesized in small lymphocytes includes a *high proportion of sequences hybridizing to repetitive DNA sequences* Since a predominantly nuclear role has been suggested for such sequences this should not be unexpected in cells which seemingly have an ex-

tremely low nucleocytoplasmic transfer of RNA. Furthermore, since hnRNA of animal cells includes from 15 to 20% rapidly hybridizing sequences [13], the observation that newly synthesized RNA of small lymphocytes includes up to 20% of rapidly hybridizing sequences confirms that these cells synthesize mainly hnRNA.

Another property of hnRNA is the presence of polyadenylic acid sequences in form of large segments attached at the 3' end of some molecules [5, 6] and of short internal segments which seem to be transcribed [12]. Not all hnRNA molecules contain poly(A). We have assayed the proportion of nuclear RNA molecules of normal human lymphocytes carrying poly(A) sequences [22]. In fractions larger than 45S RNA this proportion has been found to be around 20%. No marked difference has been found between unstimulated and PHA-stimulated lymphocytes.

One of the most interesting characteristics of heterogeneous nuclear RNA of animal cells is the presence among its molecules of a small but significant fraction with double-stranded properties [7, 9, 11, 14]. The mechanism of formation and the functional significance of these segments are presently obscure. However, evidence has been presented that many of these apparently base-paired regions originate from transcription of the so-called repetitive sites in DNA [8]. We have recently carried out a group of experiments in order to explore the synthesis and the relative occurrence of double-stranded segments in nuclear RNA of normal human lymphocytes [27]. Our results indicate that these cells do synthesize an RNA fraction with double-stranded characteristics. As much as 2% of the whole cell RNA labeled during 3 h of incubation of small lymphocytes with ^{3}H-uridine is RNase-resistant due to its double-strandedness, and this proportion does not decrease significantly by prolonging the period of incubation, at variance with that observed in other cell types [9]. The relative occurrence of dsRNA in small lymphocytes appears therefore very high, but should not be unexpected in view of the conclusion that these cells synthesize almost only heterogeneous nuclear RNA, and that the relative occurrence of double-stranded RNA in isolated hnRNA of HeLa cells has been reported around 3% [7]. In PHA-stimulated lymphocytes, in fact, which synthesize a much smaller proportion of rapidly hybridizing RNA sequences, the proportion of labeled RNA with double-stranded properties appears much lesser than that, and shows a further decrease by increasing the period of incubation [27]. Furthermore, the life time of the double-stranded segments in small lymphocytes appears much longer than in stimulated cells [27]. This is suggested by the results of experiments

showing that cold uridine chase does not affect significantly the proportion of labeled dsRNA in small lymphocytes, whereas it causes a marked decrease of this proportion in PHA-stimulated cells

Several experiments have been carried out in our laboratory to study the characteristics of hnRNA in leukemic blast cells As far as the metabolic rate is concerned, the study of several cases has disclosed the existence of a wide range of metabolic stability of nuclear RNA in these cells However, at least in part of the cell populations studied, the life time of the heterogeneous nuclear RNA may be estimated in a figure close to that of small lymphocytes [23] The fraction of RNA with S values between 35 and 55 hybridized at saturation to DNA was always found lower than that found in normal small lymphocytes [30] Since the hybridization methods used allow little hybridization of the non reiterated gene fraction, although saturation hybridization studies do not measure the true amount of repetitive sequences transcribed, this might indicate that leukemic cells are transcribing a proportion of reiterated DNA sequences smaller than that transcribed in normal small lymphocytes Furthermore, we have also observed that unlabeled rapidly sedimenting RNA from normal PHA-stimulated lymphocytes is unable to compete with labeled leukemic RNA in the same size range [30] This lack of competition presumably reflects the inability to achieve presaturation of repeated sequences from which the RNA molecules were transcribed in leukemic blast cells These results might thus imply that the heterogeneous nuclear RNA of the latter cells differs from that of normal PHA stimulated lymphocytes for including a number of copies of a markedly different family of rapidly hybridizing RNA sequences

Another difference between heterogeneous nuclear RNA of normal small lymphocytes and leukemic cells was found studying the proportion of molecules carrying poly (A) segments [24] In normal lymphocytes, as already mentioned, this proportion was never higher than 20% in hnRNA fractions larger than 45S [22] The relative occurrence of poly(A)-containing molecules in fractions of the same molecular size obtained from leukemic blast cells was up to twice as much, mainly in fractions corresponding to S values between 50 and 60 [24]

A group of investigations carried out recently were aimed to explore the synthesis of double stranded sequences in nuclear RNA of leukemic blast cells. These experiments have shown that from 0 6 to 1% of total RNA synthesized in these cells during a short period of in vitro incubation with a labeled precursor is in a RNase resistant form with double-

stranded properties [28] This double-stranded fraction is practically all of nuclear origin, and its molecules are bound to poly(A) segments in the proportion of about 25%

DNA-RNA *in vitro* hybridization experiments have also given evidence that the double stranded RNA in leukemic blast cells is at least partially formed by rapidly hybridizing RNA segments [29] After denaturation by heating, in fact, it hybridizes up to 30% to human DNA, under conditions where only RNA segments transcribed from repeated regions of DNA would be expected to hybridize This might thus imply that, since rapidly hybridizing RNA sequences extracted from leukemic blast cells are apparently different from that isolated from normal lymphocytes, dsRNA segments isolated from leukemic cells are possibly different from that synthesized in normal lymphocytes

It has been recently suggested that in tumor cells containing C type or other viral particles, dsRNA is probably of both viral and non-viral origin [14] The need for further studies on double stranded RNA in leukemic cells is shown by the results of time course experiments carried out in our laboratory These results indicate that in some of our cases the proportion of labeled RNA in double stranded form was increased by increasing the period of incubation with the labeled precursor, a result hardly expected from a component of normal heterogeneous nuclear RNA

The characteristics of heterogeneous nuclear RNA of normal small lymphocytes and of leukemic blast cells may thus be summarized as follows The average life time of the molecules of this RNA class in both types of cell is much longer than that so far reported for rapidly proliferating cells In both types of cell the newly synthesized RNA includes rapidly hybridizing sequences There is evidence, although circumstantial, that in leukemic cells these sequences are markedly different from that of normal lymphocytes In both normal lymphocytes and leukemic blast cells, the rapidly hybridizing RNA sequences are in part involved in secondary structure In fact, a significant fraction of the hnRNA appears in RNase-resistant form with double-stranded properties About one fifth of the hnRNA molecules of normal lymphocytes carries poly(A) segments, whereas this proportion is markedly higher in leukemic cells

Both cell populations seem thus characterized by the synthesis of long-living double-stranded RNA molecules The functional significance of the dsRNA in animal cells is presently obscure However, different investigators [1, 15] have reported that extremely small concentrations of dsRNA of both viral or animal origin are able to inhibit initiation of pro-

tein synthesis in *in vitro* systems The possible relevance of these observa tions to the understanding of dsRNA function in small lymphocytes and in leukemic blast cells is suggested by the remarkable failure of processing of ribosomal precursor RNA in these cells The 'wastage' of 18S RNA, as well as the accumulation of unprocessed 32S RNA in fact might be caused, in both types of cells, by an insufficient rate of protein synthesis Inhibition of protein synthesis in rapidly growing cells, induced by anti biotics or other means, brings about a series of modifications [18, 31] in the macromolecular metabolism of preribosomal RNA, giving patterns similar to that observed in small lymphocytes as well as in leukemic cells It seems thus justified to postulate that accumulation of long living dsRNA molecules is one of the critical processes limiting the growth of leukemic cells, as well as of normal lymphocytes

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Studies on Nucleic Acids in Lymphocytes of Chronic Lymphocytic Leukaemia

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Key Words Leukaemia Lymphocytes RNA synthesis Ribosomal RNA Methylation Polysomes Polyacrylamide gel electrophoresis

Abstract The resting lymphocytes of patients with chronic lymphocytic leukaemia (CLL) contain the same amount of RNA as do those of normal individuals and in terms of different molecular species as analyzed by polyacrylamide gels there is very little to distinguish the leukaemic cells However, a relatively large amount of low molecular weight RNA similar to the SnRNA found in several other tissues is present in the CLL cells The products of transcription of the leukaemic cell nucleus have been studied by the incorporation of labelled uridine and methionine The leukaemic lymphocytes show a build up and apparent delay in processing of ribosomal RNA precursor when compared to normals but studies of methylation reveal that the production of mature ribosomal RNA occurs at a normal rate The nature of the stable high molecular weight material produced is now being studied The production of proteins on the ribosome of CLL cells seems likely to be faulty as evidenced by the deficiency of active ribosomes in the leukaemic cells Studies with selective inhibitors will show whether this is due to some fault in transcription of messenger RNA

We started this work 3 years ago in the hope of explaining the apparent impairment of protein synthesis in the lymphocytes of chronic lymphocytic leukaemia (CLL) We decided to examine RNA synthesis and especially that of ribosomal RNA (rRNA) because of the well known correlation between the latter and protein synthesis The few studies on phytohaemagglutinin(PHA) stimulated leukaemic lymphocytes have indicated that there is some abnormality of rRNA processing [1, 7, 11] Further, TORELLI *et al* [12, 13] have shown a build up of high molecular weight RNA in lymphocytes of acute leukaemia

In the present study we have looked exclusively at lymphocytes not treated with PHA The lymphocytes were prepared from fresh heparin

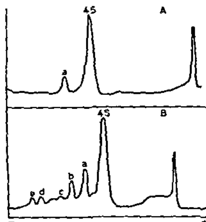
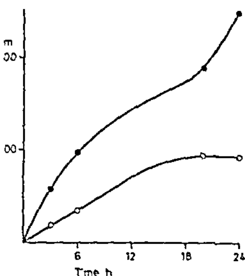
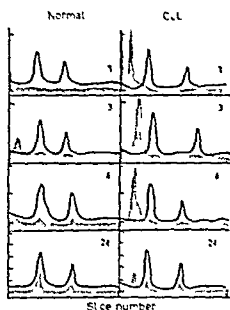


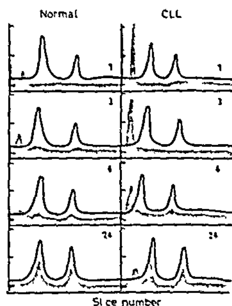
Fig 1 Scans of polyacrylamide gel electrophoresis of total cellular RNA. *A* Normal lymphocytes. *B* Lymphocytes from patients with chronic lymphocytic leukaemia Low molecular weight peaks are labelled a–e Peak a contains 5S ribosomal RNA Gels were of 5% acrylamide run for 2 h at 5 mA/gel Abscissa mobility in gel, ordinate A_{265} From Expl Cell Res 75 536–539 (1972)

ized blood by the Ficol-Triosil gradient technique [6] We have asked three questions (1) Is the amount of rRNA in CLL cells normal? (2) Is the synthesis of RNA normal? (3) Is protein synthesis in fact normal or abnormal?

Examining the first question, we measured the total RNA and DNA of the cells by the method of FLECK and MUNRO [5] We found that the ratio of DNA RNA was 2 8–2 9 1 in both normal and CLL lymphocytes We compared also the amounts of the different species of RNA Extractions of RNA were made immediately after the preparation of the cells or after storage at –20 °C, by the method of COOPER and KAY [2], and the RNA was then applied to 5 and 2 4% polyacrylamide gels, as described by LOENING [10] The results showed that the proportion of rRNA was the same in normal and CLL lymphocytes However, there was a relatively very large amount of RNA of low molecular weight, namely about 4–10S in the CLL cells (fig 1) We found these in the RNA of every patient examined (total number over 30) The RNA amounted to about 4% of the total cellular RNA, whereas with all controls the amount was less than about 0 4%. Separation of nuclei and subsequent preparation of nuclear RNA showed that these species were







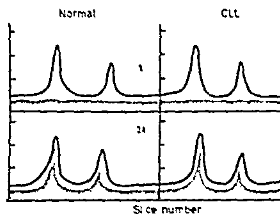


Fig 2 Incorporation (cpm) of ^{3}H uridine by cultured normal (○) and CLL (●) lymphocytes. Cells (1×10^7) were incubated in 2 ml of medium 199 with 10% autologous serum containing ^{3}H uridine (5 μCi/ml)

Fig 3 Polyacrylamide gel electrophoresis patterns of uridine-labelled RNA extracted from resting normal and CLL lymphocytes Cells were incubated for 1, 3, 6, and 24 h with 5 μCi/ml ^{3}H uridine in medium 199 with 10% autologous serum Gels were 2.4% acrylamide and run for 3 h at 5 mA/gel Scale 1 axis division equals 1 000 cpm Shaded area = cpm, — = A_{265}

Fig 4 Polyacrylamide gel electrophoresis patterns of pulse labelled RNA ex-

nuclear even though they were extractable at 0 as well as at 60 °C, unlike other types of nuclear RNA We made very careful checks to ensure that these were not degradation artefacts (1) by using a different separation method for lymphocytes – COULSON and CHALMERS' [4] defibrination technique, (2) by comparing RNA prepared from fresh and from frozen lymphocytes, (3) by omission of DNAse treatment during the RNA extraction procedure, (4) by omission of precautions against RNAase action during extraction, such as temperature control and absence of bentonite As none of these procedures affected the amount or type of RNA species in the low molecular weight region, we concluded that these species were not breakdown products of high molecular weight (HMW) RNA

To answer the second question, we examined the uptake of ^3H uridine and of ^3H methyl-methionine into the lymphocyte RNA Figure 2 shows that the incorporation of uridine is in fact higher in CLL than in normal lymphocytes However, as COOPER [3] has pointed out, it is better to examine the rate of synthesis of the different RNA species Figure 3 shows the scans of 2 4% polyacrylamide gels of ^3H uridine labelled RNA After a brief labelling period it is clear that there is a HMW RNA species in CLL which accounts for most of the label After longer periods normal cell RNA shows a higher proportion of label in the ribosomal species than does CLL RNA and by 24 h all the label is in the rRNA and none in the HMW species, in contrast, in CLL at 24 h there is still a high proportion in the HMW species We then looked at RNA after pulse labelling cells for 1 h followed by a chase with a 1,000-fold excess of cold uridine Figure 4 shows that in normal cells at 6 h there is almost no HMW all the label being in the rRNA, whereas in CLL there

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tracted from normal and CLL lymphocytes. Cells were incubated as follows 1 = 1 hour pulse 3 = 1 hour pulse+2 hour chase 6 = 1 hour pulse+5 hour chase, 24 = 1 hour pulse+23-hour chase Cells were pulse labelled with 5 μCi/ml of ^3H uridine in medium 199 with 10% autologous serum followed by a chase with a 1 000-fold excess of cold uridine Electrophoresis as in figure 3 Scale 1 axis division equals 1 000 cpm Shaded area = cpm — = A_{260}

Fig 5 Polyacrylamide gel electrophoresis patterns of pulse-labelled RNA extracted from normal and CLL lymphocytes incubated in ^3H methyl methionine (10 μCi/ml) Incubations were for 1 or 24 h in minimal essential medium without methionine (Biocult) Electrophoresis as in figure 3 Scale 1 axis division equals 250 cpm Shaded area = cpm — = A_{260} Figures 2–5 from Br J Cancer 29 318 (1974)

is still a large proportion of the label in the HMW. We then looked at methylation of rRNA after labelling the cells for 1 and 24 h with ^3H-methyl-methionine. Figure 5 shows that in 1 h there is no incorporation into either CLL or normal cells and after 24 h, the incorporation is similar into the two cell types. We conclude that the rapidly labelled HMW species is stable in CLL cells and persists for at least 24 h, whereas in normals this represents precursor rRNA which is processed into mature rRNA as usual. It should be noted that the net amount of label entering the rRNA in CLL cells is similar to that in normals – which is to be expected in view of the similar proportion of rRNA in the two cell types. This HMW RNA is probably the same species as that found in PHA-stimulated leukaemic cells by Cline [1], Henry *et al.* [7] and Rubin [11] and the present results show that it is not attributable to PHA stimulation. Also our results resemble those of Torelli *et al.* [12, 13] with actute leukaemic lymphoblasts which indicated the presence of precursor rRNA which could not be processed. Faulty processing could apply here but as methylation seems normal, possibly the precursor is normal but overproduced, or the HMW RNA is not ribosomal precursor but another species of RNA.

To answer the third question we looked at the availability of ribosomes for protein synthesis [8]. We used a method which depends on the dissociation into subunits, at higher salt concentration, of ribosomes not bound to messenger RNA (mRNA). Separation of subunits from ribosomes was then carried out on sucrose gradients and the counts were measured in the two fractions. We found that in CLL lymphocytes 16–23% of the ribosomes were active whereas in normals the values were 43–44%. This result is consistent with that of Ramsey and Ultmann [9] who used a cell-free system from CLL lymphocytes for synthesising protein from a synthetic mRNA and found that CLL cells were less efficient than normals. The two sets of results suggest that there is a fault in polysome assembly in CLL cells although there could also be a shortage of mRNA in these cells.

We conclude that in CLL lymphocytes there are abnormally large amounts of low molecular RNA species and of stable, rapidly labelled high molecular weight RNA. Methylation of ribosomal RNA and the amount of the latter in CLL cells is normal but fewer ribosomes are available for protein synthesis. It should be noted that so far in this study total normal lymphocytes have been used rather than separated B cells, for comparison with CLL; however, the abnormal RNA species in

CLL cannot be attributable only to the supposed presence mainly of B cells in this disease for the proportion of B cells in normal blood is such that much higher levels of these RNA species would then be found in normal lymphocytes than we have actually observed

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In vitro DNA Synthesis on Smooth Membranes Observed by Fluorescence

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Key Words DNA synthesis Lymphocytes Membrane proteins Nucleic acid polymerase membrane fraction RNase sensitive DNA polymerizing activity Smooth membranes

Abstract Smooth membranes have been isolated from a human diploid line of lymphocytes These membranes exhibit an endogenous DNA synthesizing capability which is partially destroyed by prior treatment with RNase In order to ascertain the role of the membranes in the DNA synthesis we have examined the conformation of the membrane proteins by observing fluorescence changes of the intrinsic probe tryptophan We have observed that on addition of the deoxynucleoside 5 triphosphates which permits DNA synthesis there are fluorescence changes due to the tryptophan residue when DNA synthesis is prevented by omitting some of the precursor triphosphates fluorescence changes are absent These effects have been observed with plasma and nuclear membrane fractions the former may contain a small fraction of the latter Similar membrane preparations from non lymphoid cells do not possess the endogenous DNA synthesizing system as shown by the lack of incorporation of radioactive precursors or fluorescence changes

A search in leukemic cells for a reverse transcriptase similar to that found in oncornaviruses has revealed that such an activity does indeed exist and that it is found associated with a membranous fraction [1–3, 7 8 11, 18, 20, 22] The latter is a high speed post mitochondrial pellet which has been characterized to the extent that it contains the endogenous RNase sensitive DNA polymerizing activity and that the DNA product hybridizes with RNAs from murine and primate viruses [2, 9] An endogenous RNase sensitive DNA polymerizing activity has also been found in normal human lymphocytes which have been transformed by phytohemagglutinin but in this case the isolated DNA polymerase is dif-

ferent from the leukemic enzyme and is not of viral origin since it does not copy 70S viral RNA [17] The reaction in this case is RNA primed and DNA-directed [17] The *in vivo* functions of either of these DNA polymerases are unknown

The present report is concerned with the nucleic acid polymerase membrane fraction in which the endogenous activity resides We have found that the endogenous activity is part of a smooth membrane fraction which is composed of plasma membrane with some nuclear membrane probably also present We also have preliminary results which show that the inner nuclear membrane contains an endogenous RNase-sensitive DNA-polymerizing activity We do not know whether the DNA polymerase in these two membrane preparations are the same To study these membrane 'complexes' in a specific and highly sensitive manner we have utilized a fluorescence technique which relies on the fact that very small changes in membrane structure confer conformational changes on membrane proteins which are accompanied by changes in the intensity of their fluorescence The tryptophan residues in proteins exhibit fluorescence when illuminated at 304 nm Since fluorescence is a function of the environment of the fluorophore (tryptophan), changes in conformation of the proteins accompanied by changes in the environment of the tryptophan can be observed We reasoned that if nucleic acids and polymerases are intimately associated with the membrane then DNA synthesis might produce conformational changes in the proteins of the membranes This expectation has been borne out We show in this preliminary report that both DNA and an RNA dependent DNA synthesis occur *in vitro* on smooth membranes from lymphocytes when they are supplied only with the deoxynucleoside triphosphates

Methods

Smooth membranes were prepared from WiL_2 a human lymphoid continuous cell line [13] Cells were swollen in hypotonic solution ruptured by Dounce homogenization, the suspension centrifuged to remove nuclei and mitochondria and the post mitochondrial pellet which contained most of the endogeneous cytoplasmic DNA polymerase activity was suspended and centrifuged to equilibrium in a sucrose gradient 5×10^9 cells were washed and suspended in 30 ml (2×15 ml) 0 01 M Tris, pH 7 4 with 0 005 M $MgCl_2$ for 5 min at 0 °C Then NaCl was added to 0 015 M and the suspension homogenized gently about 21 strokes, with a loose glass-ball pestle Nuclei were removed by centrifugation for 5 min at 1 000 g and discarded The cytoplasm was given a low-speed centrifugation at 8 000 g (15 min)

and then a high speed centrifugation at 80 000 g (1 h) The pelleted material from the second centrifugation was suspended in 3 0 ml of 65% (w/w) sucrose containing 0 01 M Tris, placed at the bottom of the tube and overlaid with 50–25% sucrose It was centrifuged at 40 000 rpm in an SW 40 rotor for 18 h The fraction at density 1 16 was removed from above with the aid of a Buckler Densiflow, collected by centrifugation and the resulting pellet suspended in 0 2 ml of assay buffer (0 04 M Tris pH 7 8 0 002 M EDTA 0 02 M $MgCl_2$, 0 04 M KCl, 0 02 M DTT) The assay mixture also contained in 0 1 ml 100 µM each of the three unlabeled precursors 1 µM ^{3}H-dGTP with a specific activity of 10 000 cpm/pmol and 100 µM ATP The reaction mixture was incubated for 30 min at 37 °C Each assay contained 10–50 µg of membrane protein For the RNase pretreatment 10 µg RNase/assay was added for 15 min at 37 °C before addition of the deoxynucleoside triphosphates The fraction in the 1 16–1 18 density range was collected, pelleted by centrifugation, recentrifuged on a similar gradient and the fraction at density 1 16 was again pelleted and resuspended Similar active fractions have been prepared from cells lyzed with Nonidet P-40

Fluorescence measurements were made with a Cary 50-026-900 differential recording spectrophotofluorometer using front surface illumination at an angle of 23° between the exciting beam and the emitted light Illuminating light is broken up by a chopper and passed alternately through a Rhodamine B quantum detector, located in the sample compartment and the sample The signal displayed on the recorder is the ratio between these two signals, thus correcting for variation of light intensity of the source Variations in monochromator transmission and photomultiplier response with wavelength are instrumentally compensated to give corrected spectra

Results

The membranes have been characterized both morphologically and biochemically Examination by electron microscopy reveals only smooth membranes with some small vesicles There is no evidence of mitochondria or ribosomes The biochemical markers for membrane fractions were 5'-nucleotidase, 0 061 µmol P/mg protein/min, monoamine oxidase, 0 00 µmol P/mg protein/min malate dehydrogenase, 0 14 OD units/mg protein/min, glucose-6 phosphatase, 0 0 OD units/mg protein/min These results indicate that there is no contamination by endoplasmic reticulum (absence of glucose-6 phosphatase), or inner or outer mitochondrial membranes (absence of malate dehydrogenase and monoamine oxidase, respectively) Therefore, most of the smooth membrane in the ρ = 1 16 fraction is probably plasma membrane, with perhaps some nuclear membrane from nuclei accidentally ruptured during cell lysis

This membrane fraction synthesizes DNA, in the presence of the four deoxynucleoside triphosphates DNA synthesis as shown by the incor

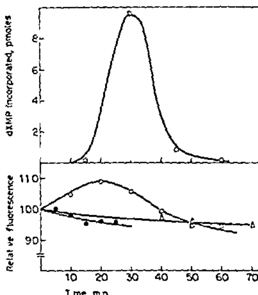


Fig 1 Lower panel The percent change in fluorescence after addition of deoxy nucleoside triphosphates to a smooth membrane preparation from WiL₂ cells in standard buffer assay ○ = Complete systems ● = minus dCTP, △ = minus dGTP The wavelength of the exciting beam was 304 nm and emission was observed at 333 nm. *Upper panel* DNA synthesis in the same system, as monitored by acid precipitable counts in the presence of all four deoxynucleoside triphosphates The values shown were obtained with ³H-dGMP but a similar curve was obtained with ³H-dTMP

poration of ³H-dGMP into acid-precipitable form, is followed by degradation (fig 1), the degradation is due to a variety of nucleases, including RNase H, present in the membrane fraction When the four deoxynucleoside triphosphates are added to the membrane fraction in assay buffer at 37 °C, fluorescence changes also occur The results are shown in figure 1 as the percent change in fluorescence (compared to that at zero time) It can be seen that there is first an *increase*, then a gradual decrease in fluorescence Since at the wavelength used most of the fluorescence is due to tryptophan we deduce that conformational changes must be occurring in membrane proteins An increase in fluorescence (a rarely seen phenomenon) indicates that the trypotphan residue enters a more hydrophobic environment, a decrease indicates a more hydrophilic environment DNA synthesis and fluorescence in figure 1 were measured in

aliquots of the same reaction mixture. In general the peak of DNA synthesis is slightly displaced from the peak of fluorescence, but when DNA synthesis is prevented by omitting dCTP or dGTP from the reaction mixture, fluorescence changes are nearly absent (fig. 1) and therefore we conclude that DNA synthesis causes the conformational changes in the membrane proteins. The small fluorescence change which is observed in the absence of dCTP or dGTP is due to a small amount of DNA synthesis which continues in the presence of only three deoxynucleoside triphosphates. The nature of this product is unknown. We calculated from our data that the ratio of DNA synthesized to protein present in the membrane fraction studied is $1:10^{11}$. That is, remarkably little DNA synthesis is required to produce measurable changes in membrane conformation.

To determine whether DNA or RNA or both are involved in the reaction, we pretreated the membranes with RNase prior to addition of the deoxynucleoside triphosphates and obtained markedly less DNA synthesis as determined by acid-precipitable counts. The decrease was variable depending on the particular preparation but it ranged from 50 to 95%. Since there was always some residual endogenous activity we tentatively conclude that both DNA and RNA can participate in the reaction. At present we are investigating whether the RNA in the RNA-dependent reaction acts as primer or template or both. Quantitative analysis of the product(s) is difficult because of the proven presence of nuclease (including RNase H) activities.

In order to learn whether smooth membranes from other sources also possess an endogenous DNA-polymerizing system, we examined membranes from SKL-N (a lymphoid line originating from a normal donor [19]) and SKL-7 (a lymphoid line originating from peripheral blood of an acute myelomonocytic leukemia [19]). We observed both RNase-sensitive and -insensitive activity in both cases. But CV-1 (an established line of monkey kidney cells), CLM-7 (a mouse fibroblast line) and Fl-1 (a human amnion line) showed no endogenous activity. These results suggest that endogenous DNA-synthesizing activity in smooth membranes may be characteristic only of lymphocytes. Since some cell types lack this activity, it seems unlikely that the binding of nucleic acid and polymerases to membrane is artifactual in the case of lymphocytes. We also examined membranes [15, 16] from adult stationary and regenerating rat liver. Neither of the latter membrane preparations exhibited the endogenous reaction as monitored by fluorescence or radioactive assay.

Table I DNA synthesis in membranes from stationary and regenerating rat liver (pmol dTMP incorporated/mg membrane protein)

	Complete systems				
	without additions	+ RNase (10 μg assay)	+ actinomycin D (5 μg assay)	+ activated Salmon sperm DNA (10 μg/assay)	+ *E. coli* Pol I (1 ng assay)
Stationary	0.21	0.14	0.11	0.75	9.5
Regenerating	0.23	0.20	0.10	4.5	17.8

For assay conditions, see Methods. Each assay sample contained 36 μg liver membrane protein.

However, on adding *Escherichia coli* polymerase I to the liver membranes, DNA synthesis did occur (table I). Therefore, we conclude that the liver fraction contains a primer-template, either RNA or DNA or both since both can be used by *E. coli* polymerase I [4–6, 10, 12, 14]. DNA polymerase is also present in the liver membrane fraction, as shown by adding calf thymus DNA together with the four triphosphates. It can be seen in table I that substantial DNA synthesis occurred. These results show that although both nucleic acid and DNA polymerase are present in these membranes, they are not accessible to each other since no endogenous reaction occurs.

Fluorescence changes occurring when polymerase I (fig. 2) is added to liver membrane preparations are markedly different from the change produced by the endogenous reaction in lymphocyte membrane (fig. 1). Only a decrease in fluorescence is observed. These results suggest that increased membrane fluorescence results only from an intimate association of membrane with an active, endogenous DNA-synthesizing system, decreased fluorescence may be related to nuclease activity in the membrane.

To show that the fluorescence changes arise from membrane proteins rather than polymerase (which in any event is present in much smaller amount), DNA synthesis in the absence of membrane was carried out with *E. coli* polymerase I, DNA and the four triphosphates. There was no fluorescence change. To test the possibility that membrane fluorescence is altered by adsorption of newly synthesized DNA to the membranes, exogenous DNA was added to the liver membrane fraction in

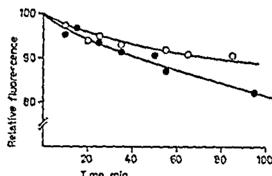


Fig 2 Fluorescence changes obtained with smooth membrane isolated from stationary (○) and regenerating (●) rat liver Conditions as in figure 1 except that *E coli* polymerase I (10 ng) was added to 1 1 ml membrane suspension

the absence of triphosphates No change in fluorescence was observed when either 2 5 or 250 μg/ml of DNA was added to the membrane Irrelevant fluorescence changes due to turbidity changes during the reaction have been ruled out by monitoring the scattering of light at 450 nm wavelength as a function of time No change was found

In addition to studying the smooth membrane fraction discussed above, we have begun a study of nuclear membranes Nuclei were prepared from WiL_2 cells using the double detergent (NP-40, DOC) method [21] which strips off the outer nuclear membrane This material showed no endogenous DNA polymerase activity, however, brief sonication of the nuclei yielded an endogenous RNase-sensitive activity in the supernatant The activity liberated by sonication has been shown to be membrane associated and is presumably on the inner nuclear membrane It is of particular interest that the nuclear pellet itself shows a marked endogenous DNA polymerase activity which is not sensitive to RNase Thus two types of activity, and perhaps two different polymerases have been separated by this simple procedure

In summary, the increase in membrane fluorescence resulting from endogenous DNA synthesis in a smooth membrane preparation from lymphocytes indicates a change in membrane configuration which must arise from a very close interaction between the membrane and the DNA synthetic system The great sensitivity of the interaction suggests a functional relationship between the components Thus for example membrane might play a role in the control of certain types of DNA synthesis The smooth membrane preparations studied contain mainly plasma

membrane, with a probable admixture of nuclear membrane The exact location of the endogenous activity, and the polymerase responsible for it, are now under study

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Mechanical Stability of Human Haemoglobins

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Key Words Haemoglobin stability Haemoglobinopathies Mechanical stability Unstable haemoglobins

Abstract The mechanical stability of oxyhaemoglobins A Agenogi, C, E, F, J Toronto, Little Rock, Riverdale Bronx, San Diego, Stanleyville-2, and Tak was found to be similar, that of A_2, Duarte and D Punjab was slightly subnormal and of Bucuresti, H Köln, M Saskatoon, S, Shepherds Bush and Zürich was markedly decreased Cyanide increased the stability of the latter haemoglobins

It has been reported [1] that oxyhaemoglobin S is denatured more rapidly than hemoglobin (Hb) A, C or F on mechanical agitation This report presents results on the mechanical stability of a number of other haemoglobin variants

Materials and Methods

Haemolysates containing 8–12 g Hb/100 ml were obtained from packed erythrocytes which had been washed 3 times in 0 9% aqueous sodium chloride solution, by addition of one volume of distilled water and half a volume of chloroform followed by shaking and centrifugation These were used without further treatment.

Haemolysate (0 12 ml) was added to tris-HCl buffer (0 1 M, pH 8 0, 12 ml) and divided into 3 equal portions of which two were placed in round bottomed Pyrex centrifuge tubes (13×105 mm internal dimensions) capped with parafilm, maintained at 37 C for 5 min and shaken on a Griffin Flask Mixer (Griffin and George Ltd., Wembley England) in a hot air room at 37 C for 15 min at two-thirds maximum speed After centrifuging (3 000 rpm for 5 min) the optical density of the supernatant was measured at 540 nm in a Unicam SP600 Spectrophotometer or at 280 nm in a Perkin Elmer Spectrophotometer and the average reading of the two portions which had been shaken expressed as a percentage of that of the unshaken

control The difference from 100% was taken as a measure of the amount of Hb which had precipitated

Normal haemolysates were stored in a refrigerator and used within 4 days of preparation Some of the haemolysates containing abnormal haemoglobins had been stored in the oxyform in liquid nitrogen for varying periods The electrophoretic and absorption properties of these did not appear to have been significantly altered by storage The oxyform of Hb A_2 H and S were prepared fresh by DEAE Sephadex chromatography [2]

The stability of Hb S was measured in the presence of each of the following to give the final concentrations shown D-glucose (0 14 M) urea (0 02 M) glycine (0 02 M) $Na_2S_2O_4$ (0 02 M) KCN (0 1 M) NaOCN (0 1 M) $K_3Fe(CN)_6$ (0 01 M) NaN_3 (0 01 M) NaF (0 01 M) L asparagine (0 007 M) L glutamine (0 007 M) L homoserine (0 007 M) The carbonmonoxy form was obtained by bubbling CO gas through the haemoglobin solution before use

Results

The amount of precipitate formed by mechanical agitation of 20 haemolysates which contained no abnormal variants on filter paper electrophoresis (pH 8 8) ranged between 4 and 20% (mean 14, SD ± 4%), while that formed by a haemolysate containing 71% alkali resistant haemoglobin (Hb F) was 8% When one of several structure variants (Hb Agenogi, C, D Punjab E, J Toronto, Little Rock, Riverdale Bronx San Diego, Stanleyville 2 or Tak) comprised 25–50% of the total haemoglobin, the rest being Hb A and normal amounts of Hb A_2 the amount of precipitate formed ranged between 5–20% With haemolysates containing Hb Bucuresti, Koln, M Saskatoon, S, Shepherds Bush or Zurich in amounts 20–45% of the total, the amount of precipitate formed was 30–45% Purified Hb A_2 H and S produced precipitate amounting to 32, 98 and 94% respectively

The precipitate of denatured Hb S was solubilized by KCN (0 03 M) 2 mercaptoethanol (0 5 M), acetic acid (0 5 M) and NaOH (0 5 M) Hb S and A in the carbonmonoxide, fluoride or azide forms had the same stability as their respective oxy forms In the presence of sodium dithionite or potassium ferricyanide, the stability of Hb S was unchanged while that of Hb A was markedly decreased

Hb A_2 in amounts between 2 5 and 7 5% of the total haemoglobin did not affect the stability of Hb S No significant change in stability of Hb S was noted in the presence of D glucose, urea, D glucose and urea glycine, L-asparagine, L-glutamine, L-homoserine cyanate, 2 mercapto

ethanol, dithiothreitol or buffered thioglycollate Cyanide decreased the amount of precipitate formed from purified Hb S by 50–70% Cyanide completely stabilized purified Hb A_2 and H as well as haemolysates containing Hb Bucuresti Shepherds Bush or Zurich Samples of chromatographically purified Hb F Jamaica, G Philadelphia, J Birmingham, Moskva, O Arab and St. Luke s, which has been stored in deep-freeze for varying periods, contained appreciable amounts of methaemoglobin, showed some trailing on electrophoresis and gave abnormal amounts of precipitate on shaking, were also stabilized by cyanide

Discussion

These results extend the mechanical stability of the oxy form of Hb A, C and F [1] to Hb Agenogi, E, J Toronto, Little Rock, Riverdale Bronx, San Diego, Stanleyville-2 and Tak, and the instability of Hb S to Hb A_2, Bucuresti, Duarte, H, Koln, M Saskatoon Shepherds Bush and Zurich Eight of these variants (i e Bucuresti, Duarte, H, Koln, M Saskatoon, Riverdale-Bronx Shepherds Bush and Zurich) are usually described as 'unstable' haemoglobins [3] and all of them except Riverdale Bronx were also found to be mechanically unstable

Several differences are known in the behaviour of haemoglobins to different forms of stress (a) Hb S is stable in the heat and the isopropanol tests but unstable on shaking, (b) Hb F is stable in the heat test, unstable in the isopropanol test but stable on shaking, (c) Hb Riverdale Bronx is unstable in both the heat and the isopropanol tests but stable on shaking, (d) the stability of Hb A, A_2 S and C at 45 °C (measured by changes in protein fluorescence) was in the order A_2>A>S = C, and A_2 modified the stability of both S and C [4, 5], but their stability on shaking was in the order of A = C>A_2>S and A_2 had no effect on the stability of S, (e) the precipitate formed in the heat and isopropanol tests is usually white in colour, while that formed on shaking is invariably reddish brown or black Studies on the denaturation of Hb A using electron spin resonance spectroscopy have revealed the formation of hemichromes (i e low-spin derivatives of ferrihaemoglobin resulting form changes of protein confor mation, such that atoms forming part of amino acid side chains become bound as the sixth ligand of haem iron) as important intermediates [6] That precipitation by mechanical stress may involve hemichrome formation is suggested by the finding that (a) hemichromes can be produced by

a great variety of denaturing agents [7], (b) formation of Hb H inclusion bodies is accompanied by hemichrome formation [8], (c) the Hbs Bucuresti, Duarte, Köln, M Saskatoon, Shepherds Bush and Zurich have substitutions in proximity to the haem iron atom and (d) cyanmethaemoglobin cannot form hemichromes However, denaturation by mechanical agitation appears to be less dependent on the oxidation state of the haem iron than on the structure of the subunits of the susceptible haemoglobins

The effect of cyanide was not unexpected in the light of previous findings that cyanide protects Hb A, Gun Hill and H against heat denaturation and prevents inclusion body formation in erythrocytes containing Hb Philly This effect of cyanide has been attributed to the increased planarity of the haem group and the increased stability of the haemoglobin complex of cyanmethaemoglobin (8)

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Monocytopoiesis in Normal Man: Pool Size, Proliferation Activity and DNA Synthesis Time of Promonocytes

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Key Words Cell kinetics DNA synthesis Monocytopoiesis Promonocytes

Abstract Monocytopoiesis was analyzed in the bone marrow of healthy individuals Promonocytes were identified by simultaneous determination of sodium-fluoride-sensitive and resistent naphthol AS-D-acetate esterases DNA synthesis activity of enzyme-positive promocytes was determined by ^{3}H thymidine (^{3}H TDR) incorporation *in vitro* DNA synthesis time of these cells was measured by a double labelling technique (^{3}H TDR *in vivo* ^{14}C-TDR *in vitro*) as well as by serial injections of ^{3}H TDR The relative number of promonocytes in the myelogram averaged 2.9% corresponding to a medullar promonocyte pool of about 600×10^{6} cells per kilogram body weight The promonocytes were classified into 4 groups on the basis of nucleus morphology type I promonocytes with small lymphocyte-like nuclei (mean frequency of occurence, F = 5%, mean ^{3}H TDR labelling index LI = 7.1% type II promonocytes with large round or oval nuclei (F = 31% LI = 9.7%) type III promonocytes with large, slightly folded nuclei (F = 51%, LI = 10.1%) type IV promonocytes with large distinctly folded nuclei (F = 13%, LI = 24.9%) LI of pooled promonocytes was 12.0% Mean DNA synthesis times of the different types of promonocytes was similar and approximated 10 h (range 6.6-13.3 h) This was true under normal conditions as well as in septicaemia

Present knowledge on human promonocytes in the bone marrow is scarce This investigations have been impeded by the difficulty of identifying these cells A valuable approach to this problem was provided by Schmalzl and Braunsteiner [14] who demonstrated that promonocytes can be recognized by a strongly positive reaction of several sodium fluoride-sensitive non specific esterases This 'monocyte esterase' can be demonstrated by cytochemical reactions with water insoluble azo dyes This allows for *a combination of the cytochemical methods for cell identification* with cell labelling techniques using microautoradiography [15] Thus proliferation activity and DNA synthesis time of promonocytes can be determined

Material and Methods

Subjects Bone marrow samples were obtained from 10 healthy 24- to 66-year-old volunteers The samples were used to determine the following parameters (1) relative number of promonocytes in myelogram, (2) frequency distribution of different types of promonocytes, (3) size of medullar promonocyte pool, and (4) fraction of promonocytes incorporating ^{3}H-TDR *in vitro*

DNA synthesis time of promonocytes was examined, using a double labelling technique (^{3}H-TDR *in vivo* and ^{14}C-TDR *in vitro*), in a 69 year-old haematologically normal woman She suffered from severe cardiac failure and gave consent to the experiment The second patient was a 76-year-old woman who was brought to the hospital in a desperate stage, cachectic and unconscious Clinical diagnosis septicaemia, severe bronchopneumonia, urinary tract infection, cardiac insufficiency, atrial fibrillation, diabetes mellitus DNA synthesis time was determined first by a double labelling technique One week later when the patient became terminal, DNA synthesis time of promonocytes was determined a second time by serial injections of ^{3}H TDR In both studies the unconscious patient showed no pain reactions to marrow aspirations

Morphology, pool size and DNA synthesis activity of promonocytes Bone marrow was aspirated from the sternum with a syringe containing 1 ml 0 5% Na_2EDTA in 0 7% NaCl The aliquot was then filtered through gauze The marrow spicules were transferred to a syringe containing 4 ml autologous serum and 24 µCi ^{3}H-TDR (specific activity 5 mCi/mM, supplied from the Radiochemical Centre, Amersham, England) To prevent floating of the marrow particles on the serum, a small air bubble was left in the syringe This air bubble allowed the sample to be mixed during the following incubation period of 30 min at 37°C Subsequently, bone marrow smears were prepared and rapidly air-dried

Marrow differential count was established by evaluating 1,000 nucleated cells on May Grunwald Giemsa stained marrow smears

Other smears were taken for demonstration of promonocytes by combined reactions of NaF resistent and NaF-sensitive naphthol AS D-acetate esterases using methods described by SHNITKA and SELIGMAN [16] and SCHMALZL and BRAUNSTEINER [14] Two incubation periods, each lasting 1 h, were used for demonstration of NaF-sensitive naphthol AS D-acetate esterase (substrate supplied from Sigma, Chemical Co) The nuclei were stained for 15–30 min with Mayer's haematoxilin In the resulting preparations, 3,000 nucleated bone marrow cells were scanned to determine the relative number of promonocytes in myelogram

Some of the preparations demonstrating the above-mentioned esterase reactions were submitted to autoradiography using Ilford L_4 dipping emulsion and 13 days exposure Subsequently, the nuclei were stained for 30 min through the film with Mayer's haematoxilin Haematoxilin was leached out of the film layer by incubating the smears for about 15 min in lukewarm water To determine LI, 1,000 promonocytes were scored Cells were regarded as labelled if they were overlayed by 5 or more grains (background range 0–4 grains per cell area, mean 1 4 grains) Promonocytes recorded in this study were classified into 4 groups according to the nucleus morphology (see *Results*) Calculation of marrow cellularity based on the following data was determined according to DONOHUE *et al* [3] (1) mean medullar erythroblast pool = 5 36 × 10^9/kg body weight (2) mean medullar pool of granulopoietic cells = 11 4 × 10^9/kg, and (3) mean medullar myelocyte

pool = 2 6 × 10^9/kg From these figures and the relative numbers of corresponding cells in the subjects' bone marrow, total marrow cellularity was determined. $pool_{total\ I}$ = (5.36 × 10^9/kg × 100%) (erythroid series %), $pool_{total\ II}$ = (11 4 × 10^9/kg × 100%)/ (granulocytic series %), $pool_{total\ III}$ = (2 6 × 10^9/kg × 100%) (myelocytes %) The mean of the three results ($pool_{total\ x}$) was used to calculate the medullar promonocyte pool $pool_{promonocytes}$ = (promonocytes %) × ($pool_{total\ x}$)/100%

Determination of DNA synthesis time by double labelling 0 1 mCi ^{3}H-TDR (specific activity 15 6 Ci/mM, Radiochemical Center, Amersham, England) per kilogram body weight were injected intravenously Bone marrow was aspirated at intervals of 60 and 120 min after injection In the septicaemia patient an additional sample was taken at 180 min Samples of about 0 5 ml of the aspirate were put into a siliconized glass tube containing 1 ml of a mixture of Na_2EDTA and plasmagel (1 70 g Na_2EDTA + 1 4 g NaCl in 100 ml distilled water + 100 ml plasmagel and 1 5 µCi ^{14}C-TDR, specific activity 58 mCi/mM, Radiochemical Centre, Amersham, England) After 10 min incubation at room temperature (about 23°C), marrow particles were transferred, using a siliconized Pasteur pipette, into a second siliconized tube containing 1 ml of the above mentioned Na_2EDTA-plasmagel mixture and 1 mg unlabelled thymidine Bone marrow cells were partly washed out of the spicules by aspirating the particles and blowing them out repeatedly (approximately 8 times) with a Pasteur pipette Remaining fragments were discarded and the cell suspension spun at 150 *g* for 5 min Smears suited for microscopic evaluation were prepared from the sediment and rapidly air-dried Subsequently, promonocytes were demonstrated by the above mentioned combined reactions of NaF-sensitive and NaF resistent naphthol-AS-D-acetate esterase

The smears were covered with undiluted Ilford L_4 autoradiograpgic dipping emulsion and exposed for 80 days Then nuclei were stained with haematoxilin as described above Some of the smears obtained from the septicaemia patient were covered with a second film layer (undiluted Ilford K_5 emulsion) which was separated from the first by a layer of celloidin using a procedure described by HARRISS *et al* [6] The second film layer was exposed for 90 days

From each sampling, 1,000 labelled promonocytes were recorded and classified according to the type of labelling (^{3}H-TDR only, ^{14}C-TDR) and according to the nucleus morphology (see *Results*) DNA synthesis time (T_s) was calculated according to the following formula $T_s = (N^{14}C/N^3H) \times t$, where $N^{14}C$ = number of promonocytes labelled with ^{14}C-TDR only or with ^{14}C-TDR + ^{3}H-TDR, respectively, N^3H = number of promonocytes labelled with ^{3}H TDR only t = time interval between injection of ^{3}H-TDR and incubation of the bone marrow sample with ^{14}C-TDR

Determination of DNA synthesis time by serial ^{3}H-TDR injections 5 mCi ^{3}H-TDR (specific activity 15 6 Ci/mM) were injected intravenously 3 times at intervals of 259 min and 287 min Bone marrow aspirates were sampled 68 min after the first injection, 14 min after the second and 17 min after the third injection A control performed prior to the first injection demonstrated that there were no labelled promonocytes left from the first examination of the patient Bone marrow smears were prepared and promonocytes were analyzed using the above-described combination of cytochemical and autoradiographic methods Ilford L_4 autoradiographic dipping emulsion was used in this study The exposure time was 79 days ^{3}H TDR labelling indices were determined by scanning 1,000 promonocytes

Table I Marrow differential counts in 10 healthy adults, %

	Mean ± SD	Range
Pronormoblast (E_1)	0.8 ± 0.2	0.4– 1.3
Basophilic normoblast (E_2)	2.4 ± 1.2	1.0– 4.5
Polychromatic normoblast, large (E_3)	4.8 ± 1.9	2.0– 7.4
Polychromatic normoblast, small (E_4)	8.0 ± 1.1	6.0– 9.4
Orthochromatic normoblast (E_5)	12.7 ± 2.3	8.4–16.3
Myeloblast (M_1)	0.9 ± 0.5	0.4– 2.0
Promyelocyte (M_2)	1.5 ± 0.6	0.8– 2.6
Myelocyte, large (M_3)	4.7 ± 0.9	3.6– 6.5
Myelocyte, small (M_4)	9.3 ± 0.8	8.1–10.8
Metamyelocyte (M_5)	10.7 ± 1.4	7.6–12.1
Band (M_6)	13.9 ± 2.3	9.8–16.6
Segmented (M_7)	9.5 ± 1.9	6.4–13.8
Eosinophil	3.7 ± 0.9	2.1– 4.8
Basophil	0.1 ± 0.1	0.0– 0.5
Promonocyte[1]	2.9 ± 0.6	2.2– 3.7
Lymphocyte	8.0 ± 2.0	5.0–11.7
Plasma cell	1.4 ± 0.4	0.6– 1.9
Reticulum cell	0.4 ± 0.4	0.0– 0.9
Megakaryocyte	0.4 ± 0.2	0.2– 0.8
Not classifiable	3.9 ± 1.5	1.4– 7.5

[1] Determined by cell identification using simultaneous demonstration of NaF sensitive and NaF resistent non specific esterases

DNA synthesis time (T_S) was calculated from the labelling index after the first (LI_1), the second (LI_2), the third (LI_3) ^{3}H TDR injection and the time intervals (t_1, t_2) between consecutive injections $T_S = (LI_1 \times t_1) \times (LI_2 - LI_1)$, respectively $(LI_2 \times t_2) \times (LI_3 - LI_2)$

Results

Morphology, proliferation activity and pools of promonocytes (fig 1) The marrow differential counts of the 10 normal individuals examined are listed in table I The relative number of promonocytes ranged from 2.2 to 3.7% with an average of 2.9% Promonocytes could be divided into 4 groups on the basis of nucleus morphology This classification was somewhat arbitrary To get an idea of cell diameters, 100 promonocytes were measured following classification This gave the follwing results *type I promonocytes*

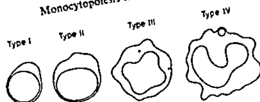


Fig 1 Morphological and functional characteristics of promonocytes in bone marrow

	Type I	Type II	Type III	Type IV
Mean cell diameter, μm	9	12	14	17
Mean frequency distribution, %	5	31	51	13
Mean ^3H TDR labelling index %	7	10	10	25

Table II Medullar pools and ^3H TDR incorporation *in vitro* (mean SD, range) of different types of promonocytes in healthy individuals

Nucleus morphology of promonocytes	Frequency distribution, % n = 10	Medullar pool cells × 10^6/kg body weight n = 9	^3H TDR labelling index, % n = 7
Small round to oval (type I)	5 3 ± 1 7 (2 8 – 8 0)	30 1 ± 11 9 (17 6 – 53 6)	7 1 ± 5 5 (2.1 – 17 1)
Large round to oval (type II)	30 6 ± 3 2 (25 7 – 35 9)	176.5 ± 26 9 (142.8 – 218 6)	9 7 ± 2.2 (7 6 – 14 0)
Large slightly lobed (type III)	50 6 ± 2 0 (47.2 – 54 4)	296 9 ± 66.2 (207 1 – 421 9)	10 1 ± 1 8 (7 1 – 12.9)
Large distinctly lobed (type IV)	13 5 ± 3 4 (9 6 – 21 8)	79.3 ± 26 7 (56 1 – 136 7)	24 9 ± 2.5 (21 7 – 28.3)
Total of pooled promonocytes		584 4 ± 117 6 (438 8 – 775 4)	12.0 ± 1 8 (9.2 – 14.2)

exhibit lymphocyte size Their nuclei are small or oval The chromatin is tight Range of cell diameter 7.2–9 8 μm, mean 8 7 μm, *type II promonocytes* are endowed with large round or oval nuclei, a fine chromatin pattern which often contains one or two nucleoli Range of cell diameter 8 5–15 8 μm, mean 12 2 μm *type III promonocytes* demonstrate large and slightly folded

Table III. DNA synthesis time of promonocytes determined by a double labelling technique and single emulsion autoradiography in a haematologically normal patient

Minutes after ^{3}H-TDR pulse labelling	DNA synthesis time, h		
	type II	type III	type IV
60	12.6	10.5	8.0
120	10.2	9.2	7.4

Table IV. DNA synthesis time of promonocytes in a septicaemia patient (first examination) determined by a double labelling and single emulsion (A) as well as double emulsion (B) autoradiography

Minutes after ^{3}H-TDR pulse labelling		DNA synthesis time, h		
		type II	type III	type IV
A	60	13.3	8.4	7.1
	180	12.3	7.0	6.6
B	60	9.3		8.0
	120	10.2		8.1
	180	9 6		14 1

Table V. DNA synthesis time of promonocytes in a septicaemia patient (second examination) determined by serial injections of ^{3}H-TDR (t_1 = interval between first and second, t_2 = interval between second and third ^{3}H-TDR injection, see fig. 1)

	DNA synthesis time, h		
	type II	type III	type IV
t_1	9.3	11.6	7.7
t_2	10.2	9.9	–

nuclei, a fine chromatin pattern. Nucleoli are scarce. Range of cell diameter 11.2–17.2 µm, mean 14.2 µm, *type IV promonocytes* demonstrate large and distinctly folded nuclei with a fine chromatin pattern. Range of cell diameter 14.5–19.8 µm, mean 16.9 µm. The mean frequency distribution of the different precursor types was: type I = 5%, type II = 31%, type III = 51%, and type IV = 13% (table II).

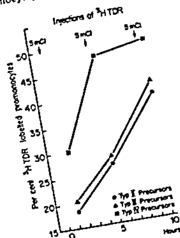


Fig 2 Determination of DNA synthesis time of promonocytes by serial ^{3}H TDR injection*

Total medullar promonocyte pool of the 10 healthy individuals averaged 584 × 10^6 cells per kilogram body weight The compartment sizes of various types of promonocyte are listed in table II ^{3}H-TDR labelling index of pooled promonocytes averaged 12% (table II) The mean labelling indices of various precursor types were type I = 7 1%, type II = 9 7%; type III = 10 1%, and type IV = 24 9%

DNA synthesis time DNA synthesis time of type I precursors could not be determined because the number of scanned cells was too low to give reliable results In smears treated by the double emulsion technique, type II and III precursors were pooled as differentiation was nearly impossible The different techniques applied (double labelling method combined either with single or double emulsion autoradiography and serial ^{3}H-TDR injections) gave similar results under normal conditions as well as in septicaemia (tables III–V) There was no significant difference of DNA synthesis time between the various precursor types Twenty-three determinations of DNA synthesis times were carried out in the two patients The values ranged between 6 6 and 13 3 h and averaged 9 5 h

Proliferating fraction of promonocytes and cell cycle time in septicaemia Figure 2 illustrates the behaviour of the promonocyte labelling indices during a series of three ^{3}H-TDR injections in a septicaemia patient The initial

labelling indices nearly doubled the figures obtained in normal individuals (table II). Labelling indices of type IV precursors exceeded the normal values only by about 20%. During the course of the study, labelling indices of type II and III precursors increased at rather constant rates. In contrast, labelling indices of type IV precursors achieved a plateau at values of about 50%. This plateau indicates the 'growth fraction'. The data evaluated in the experiment allow the cell cycle time (T_C) of the patients' type IV promonocytes to be calculated:

$$T_C = \frac{T_S \times \text{growth fraction}}{\text{initial } ^3\text{H-TDR LI}} = \frac{7.7\ \text{h} \times 50\%}{30\%} = 12.8\ \text{h}$$

Discussion

At the present, only scanty data concerning human monocytopoiesis are available in the literature. Leder [7] analyzed the monocytic series in man by combining several cytochemical methods. The relative numbers of promonocytes reported by this author ranged between 0.2 and 3.4%. Schmalzl and Braunsteiner [14] employing demonstration of NaF-sensitive non specific esterases for promonocyte identification found relative numbers of the monocytic series between 2 and 5%. The data evaluated by our study confirm these findings.

Detailed monocytopoietic studies in NSC-Swiss-mice were carried out by van Furth and coworkers. Van Furth and Cohn [4] separated cells of the monocytic series by cell attachment to glass surfaces. On the basis of morphological and functional criteria, the collected cells were grouped into promonocytes and cells designated as bone marrow monocytes. The ratio was 20:80%. Promonocytes isolated from mice had the following characteristics: cell diameters ranging from 14 to 20 μm, large and reniform nuclei, ^{3}H-TDR labelling indices of about 70% after injection of a single dose of ^{3}H-TDR, DNA synthesis time of 13.6 h and cell cycle time of 19.5 h [5]. The monocytes separated from mouse marrow displayed large and folded nuclei and did not incorporate ^{3}H-TDR.

Comparison of promonocytes in man and in mice demonstrates some substantial discrepancies: (1) small, lymphocyte-like monocyte precursors corresponding to human type I promonocytes were not detected in mice. This may be due either to non-existence of these cells in mice or to ineffective promonocyte isolation; (2) non proliferating bone marrow monocytes

were present in mice which morphologically, equalled those of human type IV promonocytes Type IV promonocytes, however, demonstrated the highest DNA synthesis activity among all promonocyte types occurring in human marrow, (3) DNA synthesis activity of mice promonocytes greatly exceeded that of human promonocytes It is assumed that cell separation by adhesiveness to glass surfaces used in mice studies may partly account for these discrepancies

It is of special interest that DNA synthesis activity of human promonocytes is relatively low under normal conditions It did not reach the level of proliferating human erythroblasts [13] or granulopoietic cells [2] Higher values of promonocyte DNA synthesis activity, however, were found in the septicaemia patient This is in accord with observations in patients undergoing surgical operations which demonstrated a rapid rise in promonocyte proliferation activity during the early phase of the inflammatory response [12] From these findings it can be derived that normal monocytopoiesis is endowed with a proliferation reserve in the proliferation pool of the system This proliferation reserve is utilized under conditions of enhanced cell demand such as infection and inflammation Several results indicate [8, 12] that the small lymphocyte like type I promonocytes possess the highest proliferation reserve

Microkinematographic observations of living promonocytes demonstrated that the motility of nuclei and cytoplasma increases with maturation time [11] This motility influences the appearance of the cells when fixed on smears Immature and slowly moving promonocytes will with higher probability, be fixed as cells with round or oval nuclei With increasing maturation the probability of the cells to be fixed with folded nuclei rises due to the enhancement of cellular motility

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Proliferation of Ineffective Erythropoiesis with Nuclear Abnormalities and Megaloblastoid Appearance in Preleukaemia

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Abstract. Erythropoiesis of two patients in the early stage of acute leukaemia and two patients with refractory anaemia and hypercellular bone marrow (preleukaemia?) was studied with the cytophotometric autoradiographic method. Megaloblastoid erythroblasts show a decreased proliferative activity in comparison to the morphologically normal cells and are in the maturation stage of E_4 (early polychromatic normoblasts) mainly in the G_1 phase, they are therefore largely comparable to the megaloblastoid erythroblasts in erythroleukaemia. Erythroblasts in preleukaemia with nuclear abnormalities are found in a high percentage in the G_2-phase or are unlabelled with a DNA content of between diploid and tetraploid value. They show a similar proliferative behaviour to the megaloblasts in pernicious anaemia. Early polychromatic erythroblasts arrested in G_2 phase can differentiate without mitosis into tetraploid mature erythroblasts (E_6). They can divide elsewhere endomitotically, produce binucleated E_6 or take up the DNA synthesis and become polyploid. E_4 with nuclear abnormalities do not proliferate and are mainly found in the premitotic phase.

Erythropoiesis in pernicious anaemia is the best studied model of ineffective erythropoiesis, the disturbance of cell proliferation has been well defined by *in vivo* labelling with tritiated thymidine as well as by cytophotometric autoradiographic investigations [1, 6, 7, 10–12]. In erythroleukaemia there is likewise an ineffective erythropoiesis [4, 8, 9]. The megaloblastoid cells represent, in contrast to the erythroblasts in pernicious anaemia as cell population mainly found in G_1-phase and do not proliferate

1 We wish to thank Miss M. Broszies for excellent technical assistance.

in the maturation stage of early polychromatic normoblasts [8] Erythroblasts with megaloblastoid appearance occur in preleukaemic states [2, 3], at the same time marked nuclear abnormalities can be seen Our cytophotometric-autoradiographic investigations answer the question whether these cell abnormalities are the result of a defined disturbance of erythropoietic cell proliferation in preleukaemic states

Methods

Single cell suspensions of bone marrow were incubated for 1 h at room temperature with tritiated thymidine (2 μC/ml specific activity 5 Ci/mmol) Smears were stained by the May Grünwald Giemsa method Cells were photographed and the cell areas marked with an 'Objektmarker' (Leitz) and a second slide The panchromatic stain was leached out and the smears were restained by the Feulgen method (hydrolysis in 1 N HCl at 60 °C for 9 min) The DNA content was measured with a cytophotometer UMSP I (Zeiss) at 560 nm Autoradiographs were prepared by the dipping method using the emulsion K5 (Ilford) as described previously [2]

Patients

In the present study we have investigated the erythropoiesis of four patients two males and two females At the time of examination all had anaemia (table I) three of them (No 2–4) neutropenia and three (No 1–3) thrombocytopenia. Bone marrow cellularity was increased in all cases The haematologic findings remained constant within an observation period of 6–11 months, after 6 months acute leukaemia (AML) developed in one case (No 1) Normoblasts were found in the blood smear of two patients (No 1, 3) The occurrence of blast cells in blood and bone marrow in two patients (No 1 2) supports the view that there was an early stage of acute leukaemia (table I, II) The morphological abnormalities of myelopoiesis and erythropoiesis of the other two (No 3 4) suggest the presence of a preleukaemic state In the bone marrow smears (table II) the erythropoiesis dominated except in one case (No 1) Erythropoiesis showed megaloblastoid changes (No 1 2) and in all cases nuclear abnormalities which were expressed especially in E_4 and E_5 (table II)

Results

The erythropoiesis of all patients showed an overproportionally high percentage of basophilic erythroblasts (table III) The basophilic erythroblasts as well as the subsequent proliferating compartment E_4 had a decreased labelling index (table IV) In the case of the first two patients

Table I Haematological data of the patients and the course of their disease (until 31 12.74)

Case No	Age, years	Hb, g/100 ml	Neutrophils/ µl	Platelets/ µl	Erythro-blasts/ µl	Blast cells/ µl	Diagnosis	Course
1	48	8.7	2,740	97	122	571	smoldering acute leukaemia	died 6 months after diagnosis AML
2	23	5.2	384	17	0	24	pancytopenia with hyperplastic bone marrow, early stage of AML?	blood transfusions, haematological data unchanged (6 months)
3	62	10.4	1,200	34	245	0	pancytopenia with hyperplastic bone marrow, preleukaemia?	haematological data unchanged (10 months)
4	73	6.5	496	135	0	0	anaemia and neutropenia, hyperplastic bone marrow	haematological data unchanged (11 months)

this result was to be explained in the first place by a high percentage of cells in the G_1-phase (table IV) This applies above all to the megaloblastoid cells (table V), which were to be found up to 83% in the presynthetic phase In the other two cases (No 3, 4) megaloblastoid cells were not present, here, nuclear abnormalities were the striking finding A high proportion of E_1–E_3 and, above all, E_4 consistent of unlabelled tetraploid cells in their case A further part had DNA values of between 2c and 4c and was unlabelled (table IV, fig 1, 2) E_4 with pathological nuclear configurations were mainly found in the G_2- or G_1-phase and did not include proliferating cells (fig. 3) A small percentage of the erythroblasts (table IV) had a DNA content far above the tetraploid value (fig. 1, 2) Among the oxyphilic and late polychromatic normoblasts of two patients (No 3, 4) we found a high percentage (7, respectively 8%) of tetraploid unlabelled cells (fig 4) Binucleated late polychromatic and oxyphilic normoblasts showed tetraploid DNA values and were unlabelled.

Table II. Morphological findings of the bone marrow. E:G = ratio of erythropoiesis to myelopoiesis

Case No	E:G	Blast cells %	Megaloblastoid cells, %			Nuclear abnormalities, %			
			E_1+E_2	E_3	E_4	E_1+E_2	E_3	E_4	E_5
1	0.2	15	25	42	62	0	3	3.5	15
2	1.0	7	18	35	38	2	0	8	16
3	5.5	*6 undifferentiated cells*	0	0	0	0	5.5	28	48
4	7.0	0	0	0	0	1.5	7	23	30

Table III. Results of the differentiation of 500 erythroblasts. $E_1+E_2=1$

Case No	E_{1+2}	E_3	E_4	E_5
1	1	0.7	1.0	1.6
2	1	0.7	1.0	1.1
3	1	0.6	2.7	3.3
4	1	0.4	0.5	0.5
Normal values (6 cases)	1	1.3	3.0	7.4

Discussion

The erythropoiesis of pernicious anaemia, the model of an ineffective erythropoiesis, is characterized by a decrease of the proliferating part of the more mature erythroblasts (E_4). Thus the early polychromatic normoblasts consist of two, morphologically identical populations, of which one does not proliferate and die intramedullary [7]. This theory is supported by the cytophotometric autoradiographic data of WICKRAMASINGHE *et al.* [10]. A second group of patients which likewise has ineffective erythropoiesis is erythroleukaemia [4, 8, 9]. The megaloblastoid early polychromatic normoblasts appearing here form a non proliferating cell population which arrest in the presynthetic phase and cannot enter DNA synthesis [8]. Megaloblastoid erythroblasts were also present in two of the *patients examined by us (No. 1, 2). In all maturation stages a high proportion* was present in the G_1-phase, which reached 71–83% in the early

Table IV Distribution of the basophilic erythroblasts (E_1–E_3) and early polychromatic normoblasts (E_4) in the various phases of the cell cycle in % >4c = polyploid cells U = unknown, unlabelled cells with DNA values of between 2c (diploid) and 4c (tetraploid)

Case No	E_1–E_3 n	G_1	S	G_2	U	S G_2	>4c	E_4 n	G_1	S	G_2	U	S G_2	>4c
1	251	56.5	40.5	2.6	0.4	14.4	0	290	47.9	46.2	3.2	2.7	11.1	0
2	239	30.0	52.5	13.0	4.1	4.0	0.4	199	50.0	36.5	8.5	5.0	4.3	0
3	200	19.0	61.0	12.5	7.0	4.9	0.5	304	38.3	16.5	31.3	13.6	0.5	0.3
4	363	31.0	18.7	27.8	22.2	0.7	0.3	296	31.5	8.0	54.7	3.5	0.15	2.3
Normal values (5 cases)	898							917						
Mean		16.2	75.3	6.6		11.3			33.0	55.5	5.5		10.0	
Range		11–22.6	73.5–77.0	3.0–13.5	≤0.9	5.5–24.5			28–47.5	46.5–66.5	5.0–6.2	≤0.9	7.8–10.7	

Table V Distribution of normal and megaloblastoid basophilic erythroblasts (E_1–E_3) and early polychromatic normoblasts (E_4) in the various phases of the cell cycle in %. U = unknown, unlabelled cells with DNA values of between 2c (diploid) and 4c (tetraploid)

Case No		E_1–E_3 G_1	S	G_2	U	S G_2	E_4 G_1	S	G_2	U	S G_2
1	all cells	56.5	40.5	2.6	0.4	14.4	47.9	46.2	3.2	2.7	11.1
	normal cells	49.5	45.4	4.4	0.7	10.3	28.2	62.8	5.1	3.9	12.0
	megaloblastoid cells	68.5	31.5	0	0	0	70.8	26.9	0.8	1.5	33.5
2	all cells	30.0	52.5	13.0	4.1	4.0	50.0	36.5	8.5	5.0	4.3
	normal cells	18.7	59.5	17.0	4.2	3.5	32.2	50.5	11.8	5.5	4.3
	megaloblastoid cells	51.6	40.0	4.9	3.5	10.5	83.2	11.2	2.8	2.8	4.0

polychromatic normoblasts. Cells without megaloblastic changes on the contrary showed an almost normal distribution in the various phases of the cell cycle. Megaloblastoid cells which occur both in erythroleukaemia as well as in preleukaemia represent therefore a cell population which cannot enter into DNA synthesis. This proliferative disturbance is not characteristic of certain haematological disease.

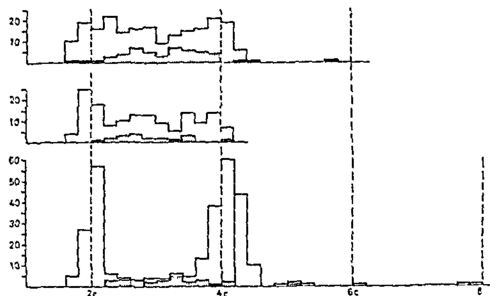


Fig. 1 Case No. 4 Relative DNA content and ^{3}H-TdR labelling of the proliferating erythroblasts Above proerythroblasts and basophilic macroblasts (E_1+E_2), middle basophilic normoblasts (E_3), below early polychromatic normoblasts (E_4) Abscissa DNA content, ordinate number of cells, white areas unlabelled cells, shaded areas labelled cells, 2c diploid, 4c tetraploid, 6c hexaploid, 8c octoploid DNA value

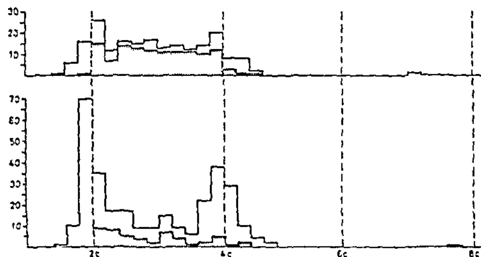


Fig. 2 Case No. 3 Relative DNA content and ^{3}H TdR labelling of the erythroblasts Above basophilic erythroblasts (E_1-E_3) below early polychromatic normoblasts (E_4). See figure 1 for legends

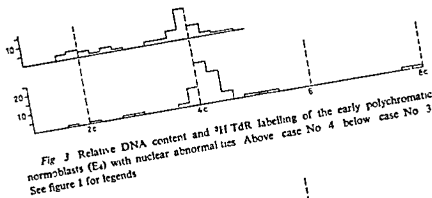


Fig 3 Relative DNA content and ^{3}H TdR labelling of the early polychromatic normoblasts (E_4) with nuclear abnormalities Above case No 4 below case No 3 See figure 1 for legends

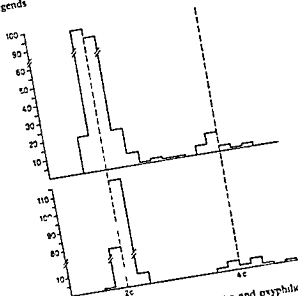


Fig 4 Relative DNA content of late polychromatic and oxyphilic normoblasts (E_5) All cells were unlabelled Above case No 4 below case No 3 See figure 1 for legends.

The analysis of the findings in the case of the two other patients (No 3 4) whose erythropoiesis is distinguished by nuclear abnormalities reveals a striking similarity to the results obtained by WICKRAMASINGHE *et al* [10] for erythropoiesis of pernicious anaemia There is a very high percentage of unlabelled erythroblasts with DNA values of between 2c and 4c as well as an accumulation of cells in the premitotic phase. It is not possible to define the further development of these cells exactly [5, 7,



Fig 5 Relative DNA content of binucleated late polychromatic and oxyphilic normoblasts (E_5) All cells were unlabelled Abscissa DNA content, ordinate number of cells, 2c diploid 4c tetraploid DNA value, above case No 4 below case No 3 See figure 1 for legends

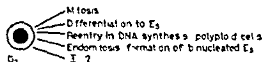


Fig 6 Possible ways of development of the early polychromatic normoblasts (E_4) arrested in the premitotic phase

10] Our results, however, show that the early polychromatic normoblasts found in the G_2-phase can differentiate without mitosis partly to tetraploid, late polychromatic and oxyphilic normoblasts (fig 4) A further way of development is to re enter DNA synthesis and produce polyploid mononucleated or binucleated cells Finally, these cells can divide endomitotically and form binucleated, mature erythroblasts (E_5) which have a tetraploid DNA content (fig 5, 6) The disturbance of erythropoietic cell proliferation described is demonstrated especially in E_4 with nuclear abnormalities These cells are mainly in G_2-phase and rarely in DNA synthesis, a small part has polyploid DNA values This finding shows that nuclear abnormalities are to be valued as a symptom of the disturbed cell metabolism and may point at a prospective cell death

For the striking disproportion between the part of E_4 present in G_2-phase and then in S phase (highly reduced S G_2 ratio) our results cannot give an exact explanation It is possible that the cells arrested in the premitotic phase remain in the bone marrow for a very long time before their further differentiation or death A part of them may, however, develop directly by differentiation from the tetraploid basophilic erythroblasts, whereby the disproportion S G_2 is simulated

The disturbance of cell proliferation in ineffective erythropoiesis can produce various morphological abnormalities of erythroblasts Morphological similarities of erythropoietic cells do not imply an identical disturb-

ance of proliferation Megaloblastoid erythroblasts in preleukaemia and erythroleukaemia are distinguished from megaloblasts of pernicious anaemia concerning the mode of the disturbance of cell proliferation, the later, however, show an identical abnormality in cell proliferation as erythroblasts with nuclear abnormalities in preleukaemic states

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Megaloblastic Changes and Chromosome Abnormalities of Erythropoietic Cells in Acute Myeloid Leukaemia

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Key Words Acute leukaemia Chromosomes Erythroblasts in leukaemia Karyotype Megaloblasts

Abstract Using Giemsa banding technique the bone marrow chromosomes were studied in 9 patients with acute myeloid leukaemia (AML) Four patients had 100% normal diploid cells and 5 had 100% abnormal cells 28-92% of the mitoses were found in erythroid cells The percentage of erythroblasts with megaloblastoid changes was abnormally high It was not increased in the cases with chromosomal abnormalities These findings indicate that chromosomal aberrations are not prerequisites for the development of megaloblasts in AML Furthermore abnormalities in the DNA synthesis bringing about the megaloblastoid changes may occur without influencing karyotype

Megaloblastoid changes of erythroblasts may result from various causes known to interfere with the normal DNA synthesis of the cells [14] In acute myeloid leukaemia (AML) various proportions of the erythroid cells may have a megaloblastic appearance [1, 3, 4 13] and biochemical abnormalities in the DNA synthesis of the erythroblasts have been demonstrated [5] Chromosomal aberrations of the erythroblasts are also present in AML suggesting that the erythroid cells may be of a leukaemic origin [2 9 10]

The present work was undertaken to investigate whether megaloblastoid changes of the erythroblasts in AML are related to chromosomal abnormalities

Material and Methods

Patients Nine adult untreated patients with a recent diagnosis of AML were studied Their age was 19 79 mean 49 years The patients were selected because

Table I Cytologic and chromosomal findings in the bone marrow of 9 patients with AML

Sex and age	Number of mitoses examined	Granulocytic or monocytic mitoses, %	Erythroid mitoses, %	Megaloblasts, %	Karyotype
F 49	120	8	92	9	normal
M 66	129	12	88	1	normal
F 62	68	67	33	12	normal
F 79	94	29	71	18	normal
F 30	148	24	76	11	abnormal
F 34	24	67	33	6	abnormal
F 68	1,000	40	58	13	abnormal
F 19	76	72	28	5	abnormal
M 19	118	28	72	3	abnormal
15 controls (mean values)	83	24	76	1 8	

chromosome analysis of their bone marrow cells by Giemsa banding had shown that all metaphases in each patient had the same karyotype, either 100% normal diploid cells or 100% aneuploid cells, and that a considerable proportion of the cells in mitosis were erythroblasts (table I)

Morphological studies Bone marrow smears were stained with May Grünwald Giemsa. In each smear 24–1,000 (average 200) cells in mitosis were examined and the proportions of mitotic figures belonging to granulocytic precursor cells and to erythroid cells were determined Through examination of 200–1 000 (average 640) erythroblasts the percentage of cells with megaloblastoid changes was registered Morphologic criteria for classification of erythroid precursors as normoblasts or megaloblasts given by HEILMEYER and BEGEMANN [7] were followed

Controls 15 apparently healthy persons served as controls Their age was 19–82, mean 49 years Morphologic studies were performed as mentioned and the proportion of erythroblasts with a megaloblastoid appearance was determined

Chromosome analysis of bone marrow aspirates by means of the Giemsa banding technique was performed as described previously [11] Whenever possible, the chromosome numbers were determined in 50 metaphases from each bone marrow preparation The number of cells karyotyped in detail by conventional staining and the G band technique were 12–34 and 7–23 respectively The detailed karyotopic data will be published elsewhere

Results

The proportions of erythroblasts with megaloblastoid changes were larger in the patient group than in the controls ($p<0\ 005$, Mann-Whitney

U test) An incrased frequency of megaloblasts was observed both in the patients with a normal bone marrow karyotype and in those with an abnormal chromosome pattern (table I) There was no apparent difference between the two groups

Discussion

The present cases of AML were selected because in each patient all mitotic bone marrow cells had an apparently identical karyotype as determined by the Giemsa banding technique Furthermore, only patients with considerable proportions of erythroid mitoses in their bone marrow were included In this way it was ensured with reasonable certainty that in each patient all mitotic erythroid cells were equipped with the same karyotype, and a morphologic comparison of the erythroblasts with a normal karyotype and those with an abnormal karyotype was thus possible

There are several indications that the erythroid cells in AML are of leukaemic origin Thus, chromosomal aberrations have been found to be common to leukaemic granulopoietic cells and erythroblasts in AML indicating that both cell types originate from a common leukaemic stem cell [2, 9, 10] This was further confirmed in 5 of the present patients

As mentioned, megaloblastic changes in the erythroblasts are generally signs of a disturbed DNA synthesis and are commonly found in AML An increased tendency to megaloblastic changes was also evident in the present cases and the frequency of this abnormality was apparently similar regardless of whether the erythroid cells had a normal or an abnormal karyotype Chromosomal aberrations are therefore obviously not prerequisites for the development of megaloblastic changes Furthermore, if abnormalities in the DNA synthesis of AML erythroblasts are causes of the megaloblastoid changes, such abnormalities may obviously occur without influencing the karyotype

It has been suggested that a defective differentiation of erythroid precursors contributes to anaemia in AML [6, 8, 12] It may be that the megaloblastic changes of the erythroblasts are morphologic signs of disturbances in the DNA synthesis of the cells leading to an ineffective erythropoiesis and that the karyotypic changes occurring in the AML erythroblasts are of minor importance for the impaired function of the erythroid tissue

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Haptoglobin, Hemopexin, Hemoglobin and Hematocrit in Newborns with Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency

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Key Words: G-6-PD deficiency · Haptoglobin · Hemopexin · Hyperbilirubinemia · Neonatal jaundice

Abstract. Hemolysis was studied in 40 G-6-PD deficient newborn infants, half of whom had bilirubin blood levels within the normal range whereas the others, who were hyperbilirubinemic, underwent exchange transfusion. Hemoglobin, hematocrit, hemopexin and haptoglobin showed no or little differences between either of the two groups and the controls. The findings confirm the authors' assumption expressed elsewhere that this form of hyperbilirubinemia is not hemolytic in nature.

In previous reports we illustrated the results obtained with phenobarbital [9] and with orotic acid [1] in preventing severe hyperbilirubinemia which often affects newborns with erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. In all the cases studied, no sign of marked hemolysis, independent of the bilirubin level, was observed. The same finding also characterized a group of jaundiced newborns, with the same erythrocyte enzyme deficiency, on whom light therapy was performed [10]. In the above mentioned surveys, the red cell turnover was estimated by controlling the hemoglobin (Hb) and/or hematocrit (Ht) values.

In the present paper the problem of the hemolytic component of this form of neonatal hyperbilirubinemia is further evaluated by means of haptoglobin (Hp) and hemopexin (Hx) determinations.

Material and Methods

Forty mature male newborns with erythrocyte G-6-PD deficiency and no blood group incompatibilities, respiratory distress, hypoglycemia or cephalhematoma and

whose mothers had received no medication potentially responsible for hemolysis during parturition, were used for this study Twenty newborns whose bilirubin blood levels ranged from 3 to 6 mg/100 ml and 20 newborns with bilirubin blood levels above 18 mg/100 ml serum composed the study group Twenty mature newborns served as controls Venous blood samples were collected from the former and the controls on the 5th day and from the jaundiced newborns immediately prior to exchange transfusion on the 4th or 5th day of life In no instance was light or phenobarbital treatment performed

Erythrocyte G-6-PD deficiency was first evidenced by the screening test of Brewer *et al* [2] In the positive newborn enzyme activity was then measured by Kirkman and Riley's method [3] Hb was determined by Drabkin s method and Ht using Ljumberg Cellocrite microhematocrit tubes Hp and Hx were determined in the serum by the single radial immunodiffusion technique as described by Mancini *et al* [8] using M Partigen s immunodiffusion plates The typifying was previously performed according to Smithies [12]

Results

The three groups studied (table I), i e (a) controls, (b) nonicteric and (c) icteric newborns with G 6-PD deficiency, were each divided into two

Table I Plasma concentration of Hp Hx, Hb and Ht values in 60 mature newborns¹

Group	Number of cases	Hp mg/100 ml	Hx, mg/100 ml	Hb, g 100 ml	Ht %
(a) Controls normal newborns	8	0	17.25 ± 9 91	18 43 ± 1 71	59 12 ± 2.29
	12	58 66 ± 33 86	14 83 ± 6 89	18 44 ± 1 54	59 00 ± 2.37
(b) G-6-PD-deficient newborns bilirubin level 3-6 mg/100 ml	8	0	15.50 ± 9 11	18 14 ± 1 60	58 62 ± 3 73
	12	67 33 ± 43 61	15 66 ± 6 91	17 82 ± 1 15	58 41 ± 2.31
(c) G-6-PD-deficient newborns bilirubin level 18 mg or more	7	0	16 85 ± 11 18	18 70 ± 1 06	60 42 ± 1 61
	13	72 46 ± 30 59	16 46 ± 9 17	17 97 ± 1 64	57 69 ± 3 17

¹ The data were obtained at the 5th day of life in the controls and in G-6-PD-deficient newborns with bilirubin level ranging between 3 and 6 mg 100 ml in the remainder with bilirubin level of 18 mg 100 ml or more immediately prior to exchange transfusion Each group has been divided into two lots in respect to the presence or absence of Hp

subgroups in relation to the presence or absence of Hp. The absence of Hp was observed in 8 controls (a) and in 8 and 7 G 6 PD-deficient newborns listed under the groups b and c.

The mean Hp, Hx and Hb values reported in table I do not evidence any significant difference between the controls and the enzyme-deficient newborns, whether the latter had hyperbilirubinemia or not. The Hp levels present an irregular distribution in all groups, which is reflected by the high SD values. The Hx, Hb and Ht levels are in all groups within a more limited range of variation. It is noteworthy that in the hyperbilirubinemic newborn infants who underwent an exchange transfusion all the parameters considered showed no significant change in respect to the others.

Discussion

Newborn infants with G 6-PD deficiency who develop severe jaundice in the absence of blood group incompatibilities or any other stressing factor is very frequent in our general casuistry concerning neonatal hyperbilirubinemia. In many hundreds of jaundiced newborn infants with this enzyme defect during the last 4 years of systematic controls, no severe hemolysis had occurred.

Analysis of the data in the present paper focuses attention on the following points. Both in normal subjects and in G 6-PD-deficient newborns, Hp were absent or practically undetectable in 23 out of a total of 60, i.e. in 38.3% of the cases. Absence of Hp on the 1st day of life in the study of LUNDH *et al.* [5] resulted in 15 out of 39 healthy newborn infants with practically the same frequency as in ours. This fact together with the irregular serum levels should lead to the conclusion that Hp determinations are not useful to evaluate the occurrence of hemolysis in newborn infants. There is, however, no doubt that when severe hemolysis occurs as for example in Rh incompatibility, Hp are always depleted [11].

The findings support the theory we previously enunciated [9] and which has been confirmed by Greek authors [6] that neonatal hyperbilirubinemia encountered in erythrocyte G 6 PD deficiency is not depending on hemolysis and bears a close resemblance to jaundice due to transiently impaired liver function as in premature babies. This assumption does not agree with many reports in the literature which have contributed to the statement that severe jaundice in G 6-PD deficient newborn babies is the consequence of a severe reduction of the red cell mass. The generalization

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Radioassay of Serum Folate with Use of Pig Plasma Folate Binders[1]

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Key Words. Carrier protein of folic acid · Folate binding · Pig plasma · Serum folate determination

Abstract. Pig plasma has been used as a source of specific folate binders for the development of a rapid radioassay to measure the concentration of folate in serum. The assay uses ^{3}H-pteroylglutamic acid as tracer and N-5-methyltetrahydrofolic acid for the construction of the standard curve. The assay is run in a two-step incubation system of 15 min each at room temperature. Comparison between the results obtained with this method and a microbiological one indicated that for sera with relatively high folate levels there was a good agreement between the two methods, whereas for sera with low folate levels the present method produces lower values than the microbiological one. The diagnostic value of the results obtained by the two methods is discussed on the basis of the availability to the tissues of the folates which are firmly bound to specific folate binders of human serum.

Studies performed in this laboratory have shown that among the plasma of a large variety of species only that of large pigs contains unsaturated specific binders for folates [9, 10]. Specific folate binders have been found also in the milk of several animal species [2, 3, 8, 12] and man [12]. Pig plasma was used as the source of folate binders for the determination of human serum folates in an assay, based on the competitive binding protein technique [10]. In this preliminary work ^{14}C-N-5-

[1] Supported by a grant from the International Atomic Energy Agency, Contract 1066/RB.

Table I Comparison of folate concentrations determined by radioassay of whole serum *and serum extracts*

Sample	Folate concentrations ng ml	
	serum	serum extract
1	9 6	10 0
2	2 1	3 4
3	12 6	14 2
4	9 7	9 5
5	4 0	6 5
6	3 4	4 8
7	1 1	2 7
8	7 6	8 4

Table II Reproducibility of the method

Sample	Number of estimations	Mean folate ± SD ng ml	CV[1] %
1	6	8 5 ± 0 40	4 7
2	6	3 4 ± 0 27	7 9

[1] CV = Coefficient of variation

teratoma of the testicle with peritoneal metastases, with severe anemia (Hb 7 9 g%) and slight macrocytosis (MCV 95 μm^3)

Discussion

In this report a rapid radioassay for serum folates is described which uses the easily available pig plasma as the source of specific folate binders and ^3H PGA in a two-step incubation system at room temperature MTFA has been used as a reference for the standard curve since this is the main human serum folate [6] It is obvious that the different results obtained from the present method and those from a microbiological one encountered in sera with low folate levels, should cause considerable concern since the determination of folate in these sera is most impor-

tant Therefore, it has to be decided which method produces the most reliable results

It has recently been established that a fraction of human serum folates is bound to proteins [11] and that part of this fraction is firmly bound to a specific binding protein [15] This fraction possibly cannot be determined by the radioassay technique but it could be used by *L casei* This may explain the above mentioned difference between the results of the two methods This explanation which has been proposed also by others [7], is further supported by the increased folate values obtained when serum is substituted by serum extract If the observed difference is due to this, it should be determined whether this specific protein which strongly binds folates promotes or inhibits their uptake by the cells Evidence has been presented showing that the function of human serum specific folate binder is not to promote folate uptake Thus, in HeLa cell monolayer culture ^{3}H-PGA bound to folate binder in folate deficient human serum was not available for uptake or delivery to the cell [16] Furthermore, addition of pig plasma to L 5178Y cell culture medium in amounts such as to be partly saturated by the PGA of the medium, produced a decrease in the number of living cells This decrease was compensated completely, and the figures became similar to those obtained in cultures lacking pig plasma when an excess of PGA was concurrently added to the medium [unpublished data]

These findings suggest that specific folate binders inhibit rather than promote the uptake of folates by the cells, and hence the bound folates cannot be easily used by them Therefore, radioassay should give results of better diagnostic value for human serum folate level than the microbiological method This is further supported by the observation of KAMEN and CASTON [7] that there is a better correlation between the hematologic findings and the folates measured by a radioassay than the level of folates obtained by a microbiological method especially in some cases in which increased concentration of human serum folate binders occurs

Another factor which can influence the results of a radioassay which uses as reference substance the *d l* MTFA is the possible different affinity of its two stereoisomeric forms Thus although it has been found that folate binders of pig plasma [10] as well as those of cow's milk [14] react with both stereoisomers of *d l* MTFA this does not imply necessarily that both forms have the same affinity Therefore a relatively low affinity of the nonphysiological form of MTFA will produce falsely higher

values This factor and/or other factors may compensate for the effect of serum folate binder in sera with relatively high folate level and thus the better correlation obtained for such sera can be explained

The relatively high level of unsaturated specific folate binders found in a patient suffering from teratoma of the testicle with peritoneal metastases can be ascribed either to the existence of a saturated serum folate binder which became unsaturated and therefore was revealed after the use of the bound folates, or to the release into the blood stream of a fol ate binder due to the destruction of a tissue In connection with the last possibility the existence of unsaturated folate binders in the lysate of granulocytes from some patients with chronic myelogenous leukemia [13] should be pointed out

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Virus-Bearing Plasma Cells in Peripheral Blood of a Patient with 'Hairy Cell' Leukemia

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Key Words Electron microscopy Hairy cell leukemia Leukemia Oncogenic virus A Plasma cells Virus in leukemia

Abstract Electron microscopic examination of peripheral blood from a patient with hairy cell leukemia revealed classical hairy cells atypical lymphoid cells and numerous pathological plasma cells Osmiophilic granular material coated the cell surface of hairy cells and lymphoid cells but not the plasmalemma of the plasma cells The most important features of the plasma cells were cytoplasmic protrusions and masses of oncogenic virus A particles in their endoplasmic reticular cisternae

In the course of systematic electron microscopic studies of leukemias we observed in peripheral blood of a patient with hairy cell leukemia pathological plasma cells bearing numerous oncogenic virus like particles These findings hitherto not reported in the literature, are described

Case Report

A 60-year-old female was admitted to the Poliklinik [1] Department of Medicine University Hospital of Zurich on April 10 1975 with anemia of unknown cause Except for pleuropneumonia in 1927 and cold tuberculous abscess in 1928 the patient enjoyed good health until autumn 1974 when she noticed an increasing fatigue An iron resistant anemia was found Clinical examination disclosed no significant abnormalities except for a slightly enlarged spleen palpable by deep inspiration Lymphadenopathy was not present

[1] We are grateful to Prof W SIEGENTHALER for kindly supplying us with blood samples and relevant case data

Laboratory findings Hemoglobin concentration 10 9 g/100 ml, leukocytes 2 900–3 600 with 55–66% normal lymphocytes and 9–12% 'hairy cells', platelets 55 000–80 000 Plasma cells were not noted

Bone marrow obtained by sternal and crista punctions was hypocellular and contained slightly decreased numbers of megakaryocytes and about 12% 'hairy cells'. Remaining cells were normal

Serum electrophoresis, bilirubin, blood sugar, cholesterol uric acid potassium, quick test, alkaline phosphatase, transaminases Wassermann's reaction and urine tests as well as X ray of lung skeleton and ECG showed no significant abnormalities

Material and Methods

For separation of the lymphocytes blood of the untreated patient was processed as follows (1) defibrination by stirring for 10 min at room temperature (2) fourfold dilution with phosphate-buffered saline (pH 7 2) containing 1% bovine serum albumin, (3) layer 6 ml of this blood solution on top of 2 ml of a Ficoll (Pharmacia Fine Chemicals Uppsala Sweden) – Hypaque (Winthrop Surbiton upon Thames Surrey, England) gradient (density 1 08), (4) centrifugation for 20 min at 2 000 rpm and 4 °C, (5) collection of the lymphocytes from the interface with a Pasteur pipette Thereafter the cells were fixed in 2 5% glutaraldehyde buffered with sodium cacodylate for 2 h at 4 °C followed by an osmification in 1% buffered osmiumtetroxide Dehydration was carried out with graded ethanol and embedded with Spurr embedding medium Thin sections were doubly stained with uranyl acetate and lead citrate and examined in a Philips EM 201 and 300 respectively

Results

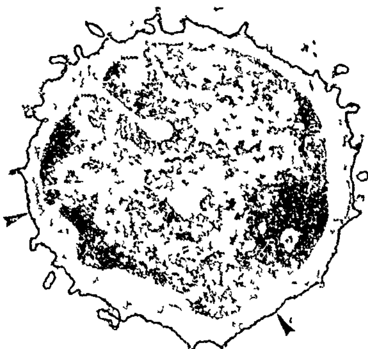
Apart from some normal shaped lymphocytes and slightly deformed erythrocytes three types of cells were observed They were clearly discernible from each other (a) Lymphoid cells (5–7 μm in diameter) with a rounded often indented nucleus, a small rim of cytoplasm with sparse cytoplasmic organelles, numerous free ribosomes and perinuclear microfilaments (fig 1–3) The cell membrane invariably formed numerous short finger-like protrusions The cell surface was regularly coated with an osmiophilic granular material of variable thickness (fig 3 arrows) (b)

Fig 1 Low power micrograph of lymphoid cells (L) and hairy cells' (H) 4 700

Fig 2 Low power micrograph of L and a plasmocyte (P) The latter contains virus like particles (arrow) Arrow heads electron dense coat on the lymphoid cell surface × 5 500



Fig 1 and



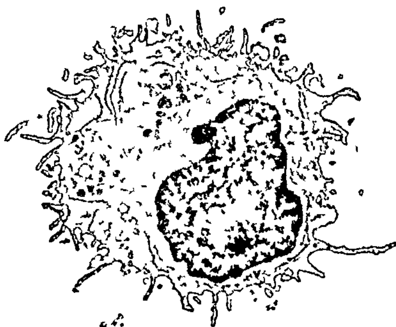
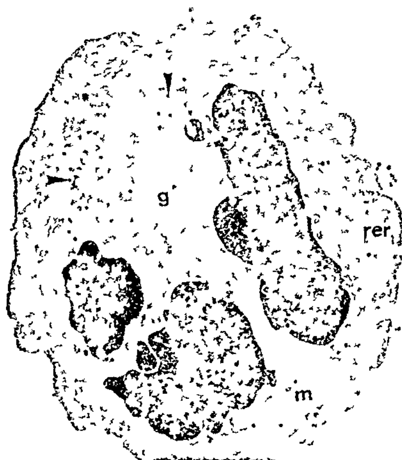


Fig 3 Lymphoid cell showing small cytoplasmic protrusions The electron dense coat on the cell surface is clearly visible (arrow heads) × 11 600

Fig 4 Classical hairy cell with numerous slender filopodia × 14 800



F g 5 Plasma cell w h an accumulat on of v rus l ke part cles (arrow heads) abundant rough end pla m c ret ulum (rer) and a large Golg apparatus (g) m = M tochondria Note the f nger and club-l ke cytoplasm c protrus ons. × 10 800

Classical hairy cells (12–15 μm in d ameter) with a large indented or lobulated nucleus in wh ch the chromatin was condensed particularly at the periphery A large nucleolus was observed in many of these nucle (f g 1 4) The cytoplasm was more abundant as compared to the lymph oid cells Stacks of few but elongated granular reticular cisternae were found at the periphery of the cell The mitochondria were inconspicuous

A well-developed Golgi apparatus with numerous vesicles and dense bodies was observed in a juxtanuclear position. Free ribosomes were present in large numbers and small lipid droplets were occasionally noticed. The most striking feature of these cells, however, was the presence of numerous cytoplasmic protrusions rendering the cell its 'hairy' impression. The projections varied considerably in length (up to 5 μm) and shape. Although most of the filopodia were slender, club-like and irregular forms also occurred. The cell surface revealed an electron dense coat similar to that described in lymphoid cells. (c) Plasma cells were present in all the specimens. They measured up to 15 μm in diameter. The main characteristic of these cells was the abundance of rough endoplasmic reticulum (fig. 2, 5). The ergastoplasmic cisternae were generally of uniform width and contained a material of moderate electron density. Furthermore, the cisternal lumina contained virus A like particles measuring approximately 75 nm in diameter (fig. 7). The particles were accumulated in some areas of the ergastoplasm (fig. 2, 5, arrows). All stages of particle buddings were observed (fig. 6, 7). Besides the membrane bound ribosomes, numerous free ribosomes and polysomes were scattered throughout the cytoplasm. The mitochondria were inconspicuous and the well developed Golgi apparatus was frequently found in a nuclear invagination. The nucleus was large, indented or lobulated. Its chromatin showed massive clumping along the nuclear membrane and around the nucleoli. The cell contour was generally smooth, however, finger- or club-like protrusions were not infrequent. Contrary to the lymphoid cells and the classical hairy cells, the plasmocytes did not show any coating of their plasma membrane.

Discussion

Hairy' cell leukemia is a neoplastic disease characterized by the appearance of cells with peculiar cytoplasmic protrusions in the peripheral blood [11]. Except for filopodia, hairy cells do not show any constant morphological features typical for a specific cell type. Hairy cells were

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Fig. 6. Virus A particles in the cisternal lumen of plasmocyte rough endoplasmic reticulum. m = Mitochondrion; g = Golgi apparatus; f = free ribosomes. × 58,500.

Fig. 7. High power micrograph of virus A particles in a plasma cell reticular cisterna. × 138,000.

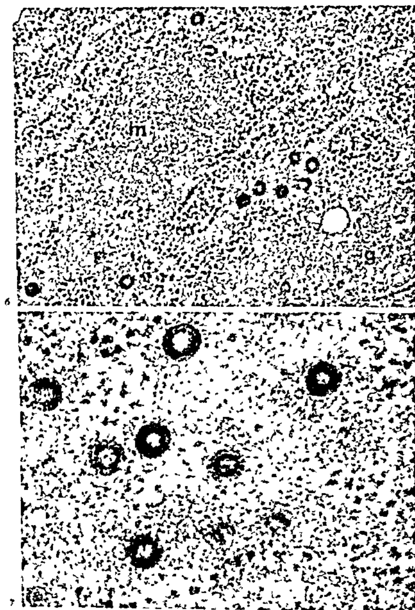


Fig 6 and 7

supposed to be (a) abnormal lymphocytes [5, 7], (b) reticulum cells [3, 9], or (c) histiocytes [2]

In the peripheral blood of our untreated patient – besides lymphoid cells and classical 'hairy cells' – numerous pathological plasma cells were found The lymphoid cells correspond to type II and the classical hairy cells' to type I of the cells described by BURNS *et al* [4] The osmiophilic granular coat of unknown origin on the cell surface of the lymphoid and the classical 'hairy' cells may be an artefact. The fact that plasmocytes never showed this coat might, however, be of some significance and might further suggest a difference in the structure of the cell membranes

The plasma cells with the constant width of the RER cisternae and the large number of free ribosomes are typical for neoplastic plasma cells as first described by DALTON *et al* [6] for murine plasmocytomas. Another important feature of the plasmocytes was the formation of occasional cytoplasmic protrusions Except for these protrusions no further common criteria between lymphoid cells, hairy cells' and plasmocytes could be found The question whether plasma cells and hairy cells belong to the same cell line or not, cannot be answered

The virus A particles constantly present in all the plasma cells belong to a class of oncogenic viruses, namely Thylaxoviridae [12] However, no evidence for a biological activity of these viruses was gained until now Generally, they are considered as precursors of mature viruses [1] Virus A particles are invariably present in murine tumor plasma cells but neither in normal murine nor in human plasmocytes [5 8 10] The presence of virus-like particles in tumor plasma cells in a case of hairy cell leukemia is suggestive for a viral nature of this neoplastic disease On the other hand, these viruses might represent 'passenger' viruses or nonspecific cell reactions

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Acute Lymphoblastic Crisis in a Patient with Chronic Lymphatic Leukemia

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Key Words B lymphocytes Immunofluorescence Lymphatic leukemia Lymphoblastic crisis Null cells T lymphocytes

Abstract A case of chronic lymphatic leukemia terminating in a lymphoblastic crisis is described The small lymphocytes were demonstrated to be B cells, they carried immunoglobulins on their surface and formed EAC rosettes The lymphoblasts had no immunoglobulins on their surface and only 18% of them formed EAC rosettes none formed E rosettes The lymphoblasts could be either immature B cells or Null cells

Chronic lymphatic leukemia is rarely complicated by acute lymphoblastic crisis Acute lymphoblastic leukemia was reported during the course of chronic lymphatic leukemia in only 4 patients [1–3] and it has been suggested that in such cases there are two distinct and unrelated diseases [1]

This report describes a patient with B cell chronic lymphatic leukemia who died in acute lymphoblastic crisis

Case Report

A 58 year-old married woman was admitted to the hospital because of weakness and low grade fever of 3 weeks duration 15 years previously chronic lymphatic leukemia was diagnosed She received no treatment and was followed up in the out patient department In the following years the number of leukocytes increased from 20 000 to 100 000 with 70-90% of the cells being small lymphocytes with a few prelymphocytes No blasts were observed On admission the physical examination

revealed a well nourished woman There was widespread lymphadenopathy The liver was palpable 10 cm below the right costal margin and the spleen 6 cm below the left border

Hemoglobin 11.0 g%, WBC 110000/μl with 39% mature small lymphocytes and 54% blast cells Platelets 43000/μl The liver tests were normal The bone marrow examinations on the day of admission revealed a heavy infiltration with lymphoblasts which constituted 80% of the nucleated cells and 10% of the cells were small mature lymphocytes The lymphoblasts (fig 1) were large cells, 13–15 μm in diameter with large vesicular nuclei and one or two nucleoli, the cytoplasm was often abundant and strongly basophilic

The diagnosis was made of acute lymphoblastic leukemia superimposed on chronic lymphatic leukemia Treatment consisted of 150 mg of prednisone and 300 mg of allopurinol daily with weekly intravenous injections of 2 mg of vincristine After the fourth injection of vincristine she suddenly developed paresis of both legs accompanied by severe paresthesias She died 5 days later from gram negative sepsis complicating a severe urinary tract infection

Methods

Two ml of heparinized blood were mixed with an equal volume of physiological saline and carefully layered upon 3 ml Lymphoprep (Nyegaard & Co., Oslo Norway) in 12 ml test tubes Centrifugation was performed for 30 min at room temperature at exactly 400 g at the interface between blood and Lymphoprep [4] This resulted in a band at the interface consisting of lymphoblasts, small lymphocytes and some monocytes These cells were harvested with a Pasteur pipette and were washed 3 times with Hanks balanced salt solution (HBSS) The fractionation of lymphocytic cells was done on a discontinuous albumin gradient [5] The cell suspension was centrifuged at 4 C at 400 g for 10 min The packed cells were resuspended in 9 parts of 33% bovine albumin solution in HBSS One ml of the cell mixture was placed into the bottom of centrifuge tubes and carefully overlayered with 1.0-ml quantities of 29 26 23 and 0.5 ml of 10% albumin solutions Centrifugation was carried out at 4 C at 20000 g for 30 min in a Sorval RC2 B centrifuge using HB4 rotor Four discrete bands of cells, which were formed at the interface between albumin solutions, were carefully harvested with a Pasteur pipette In the uppermost A band 96% of the cells were blast cells in the lowest D band 95% were small lymphocytes, and in the two intermediate bands both populations of cells were found Blast cells from A band and lymphocytes from D band were tested separately for the presence of immunoglobulins on their surface and for the formation of E and EAC rosettes

The direct immunofluorescent method was employed for the study of membrane bound immunoglobulins [6] Fluorescein isothiocyanate-conjugated antiserum to human IgG IgM and IgA (Hyland Laboratories) was used in the final dilution of 1 4 500 lymphoid cells were counted

E and EAC rosettes were prepared with sheep red cells (SRC) using the method of STJERNSWÄRD *et al* [7] with some modifications

E rosettes SRC were stored in Alsever's solution at 4 °C and used within the week of bleeding Before use the cells were washed twice and adjusted to a 1% suspension in HBSS 0 25 ml (about 10⁶) lymphocytes were mixed with 0 25 of SRC and incubated at 37 °C for 15 min The mixed cell suspension was spun at 200 g for 5 min and then incubated at 4 °C for 2 h Most of the supernatant was sucked off and the pellet was resuspended by gentle rotation of the test tube One drop of the cell suspension *was mounted onto a glass slide covered by a coverslip and sealed* with nail polish

EAC rosettes 5 ml of a 5% solution of SRC were incubated for 30 min at 37 °C with 5 ml amboceptor (rabbit anti SRC) diluted 1 2,000 in *PBS The cells were* washed 3 times and resuspended in 5 ml of PBS 5 ml of human complement (fresh human serum) diluted 1 20 in PBS were added and the suspension was incubated for 30 min at 37 °C The cells were washed 3 times and adjusted to 1%

Peripheral blood leukocytes and bone marrow cells were *stained for periodic* acid Schiff (PAS) reaction [8] α naphthyl acetate esterase [9] naphthol AS-D-chloracetate esterase [9] peroxidase [10] and acid and alkaline phosphatases [11]

Results

Immunological studies are summarized in table I 94% of small lymphocytes and none of the blast cells stained positively for surface immunoglobulins 5 6% of small lymphocytes but no blast cells formed spontaneous E rosettes with SRBC 42% of small lymphocytes (fig 2) and 18% of blast cells formed EAC rosettes (fig 3)

Cytochemical studies 20% of the blasts and all the small lymphocytes gave positive PAS reaction Staining for peroxidase, acid and alkaline *phosphatases*, naphthol-AS D chloracetate esterase was negative in the blasts and in the small lymphocytes, while α naphthyl acetate esterase was weakly positive

Table I The percentage of blasts and small lymphocytes which formed E and EAC rosettes and had Ig on their surface

	Surface Ig	Rosettes	
		E	EAC
Blasts %	0	0	18
Lymphocytes, %	94	5 6	42

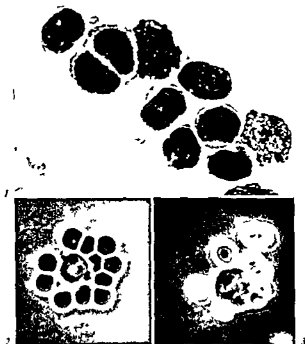


Fig. 1. Lymphoblasts in the bone marrow. May-Grünwald-Giemsa stain × 1 000

Fig. 2. Small mature lymphocyte forming EAC rosette. Phase contrast microscopy × 1 000

Fig. 3. Lymphoblast forming EAC rosette. Phase contrast microscopy × 1 000

Discussion

The occurrence of acute lymphoblastic leukemia complicating chronic lymphatic leukemia is extremely rare and we could find only 4 well-documented cases in the literature [1–3]. Of special interest are the two cases reported by BROUET *et al.* [3]: the lymphoblasts carried on their surface the same monoclonal IgM as the small lymphocytes. In both types of cells the surface IgM was proved to be synthesized *in vitro*. Moreover, in one case the IgM with anti-IgG antibody activity was found on the surface of small lymphocytes and lymphoblasts.

Our patient was known to have been suffering from chronic lymphatic leukemia for 15 years before the onset of acute lymphoblastic leukemia. She received no treatment for the chronic leukemia. The small lymphocytes showed immunoglobulins on their surface and formed EAC rosettes characteristics of B lymphocytes. The blast cells had the morphology of lymphoblasts. Their positive PAS staining, negative peroxidase, acid and alkaline phosphatases and negative naphthyl-AS-D-chloroacetate esterase reactions were diagnostic of lymphoblasts. We could not demonstrate any immunoglobulins on their surface, but 18% of the blast cells formed EAC rosettes indicating the presence of receptor sites for C3. The lymphoblasts could be either primitive B blasts not yet mature enough to have developed immunoglobulins on their surface, although some of them had already receptors for C3, or Null lymphoblasts. Null lymphocytes are believed to form EAC rosettes, but they do not carry immunoglobulins on their surface. ROSS *et al.* [12] reported that there was not always a good correlation between the presence of surface immunoglobulins and the ability of human leukemic lymphocytes to form EAC rosettes. Interestingly enough, almost all the cases of acute lymphatic leukemia have been reported to be of T lymphocyte origin [13–15], the only exceptions being the case of GAJL PECJALSKA *et al.* [16], who had no previous history of chronic lymphatic leukemia, and the two patients described by BROUET *et al.* [3], who supervened on B cell chronic lymphatic leukemia. It is therefore impossible to state whether in our case the small lymphocytes of chronic lymphatic leukemia and the lymphoblasts were derived from the same clone of cells, were two completely unrelated phenomena, or were induced by a common pathogenic factor.

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Anti-IgA Antibodies in Two Brothers with Selective Serum IgA Deficiency

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Key Words Anti IgA antibodies IgA deficiency Immunoglobulins Transfusion reactions

Abstract A selective serum IgA deficiency with anti IgA antibodies at high titer were found in a patient who suffered severe transfusion reactions The same abnormalities were detected in his brother, a 72 year old man in good general condition Anti IgA antibodies were found in the IgG fraction and were directed against α-chains A deficiency of isoagglutinins and an absence of heteroagglutinins in the propositus' serum could be shown

Strober *et al* [42] first described antibodies to IgA in patients with ataxia telangiectasia lacking IgA Subsequently, Fudenberg *et al* [15] detected anti-IgA antibodies also in serum of apparently healthy individuals without IgA This finding has important clinical implications because these antibodies can cause severe reactions following administration of blood or blood products [3, 16, 24, 28, 29, 36, 44, 46]

This paper presents our observations on a patient with a kidney tumor and iron deficiency anemia, who experienced anaphylactic transfusion reactions, afterwards we could demonstrate in his serum anti-IgA antibodies with a selective absence of IgA Some reports [2, 18, 19, 29, 31] indicate an inherited basis for IgA deficiency, studying our patient's family we detected the same abnormalities in the brother's serum

Case Reports

The propositus B B was a 77 year-old man affected by kidney tumor In the past he had been in good health except for a prostatectomy at the age of 75 He had no history of transfusion or any known allergy

Because of his low haemoglobin, a transfusion of whole blood was given but after a few milliliters of blood the patient felt epigastric pain, dyspnea and presented generalized flushing with severe hypotension. He was immediately treated with pressor amines and hydrocortisone, the recovery was slow. After a week he received a second blood transfusion to which he reacted in the same way. Immunoelectrophoresis and radial immunodiffusion revealed no IgA in the patient's serum. During the following weeks, the patient progressively deteriorated and died about 2 months after admission. Post mortem examination showed the presence of hypernephroma in the right kidney.

The brother B. L. is a 72 year-old man in good general condition. His only complaints, in the last 10 years, consisted of repeated (5) mild upper respiratory infections. He had never received blood products. No IgA were detected in his serum.

Materials and Methods

Analytical electrophoresis was carried out on cellulose acetate paper, 0.075 M tris-barbital sodium barbital buffer, pH 8.6, 250 V for 20 min. Immunoelectrophoresis was performed following the micromethod of SCHEIDEGGER [35]. Double diffusion tests were performed according to the method of OUCHTERLONY [32].

Levels of serum immunoglobulins (G, M, A and D) were determined on Tri- or Lc Partigen plates (Behringwerke) – sensitivity low as 0.01 mg/ml – according to MANCINI *et al.* [25]. IgE concentration was performed by radioimmunoassay following GLEICH *et al.* [17] using Phadedas kit.

Hemagglutination tests employed formalinized O Rh positive red blood cells [21] incubated with the antigen in the presence of 4‰ glutaraldehyde, according to SENET and PILLOT [37]. Hemagglutination inhibition tests were performed following MEYNELL's method [27].

Test antigens included: normal IgG, obtained by chromatography on DEAE-cellulose [39]; IgM from normal serum pool and from Waldenstrom's disease, isolated by cellulose acetate block electrophoresis and gel filtration on Sephadex G 200; 7 IgA myelomas (5 A_1 and 2 A_2) and IgA from normal serum pool, purified according to FINE and STEINBUCH [12] by precipitation with ammonium sulfate and caprylic acid, followed by column chromatography on DEAE-cellulose. All antigens were evaluated for purity by immunoelectrophoresis and immunodiffusion on Lc Partigen plates.

Chromatographic separation of anti IgA activity was obtained by Sephadex G 200 gel filtration of sera in 0.1 M Tris buffer, pH 8, 0.15 M NaCl. The first peak (IgM) and the second (IgG) were pooled and concentrated to the original volume of the sera. The IgM and IgG levels were estimated and the two samples were tested against the IgA-coated red cells.

Milk proteins were prepared according to LEIKOLA and VYAS [23] removing the lipids by centrifugation and precipitating casein by lowering the pH to 4.2 with acetic acid, followed by concentration to a protein content of 60–80 mg/ml and dialysis against 0.15 M Tris saline, pH 7.2.

Macroglobulins from animal sera (cow and horse) were obtained by Sephadex

Table I Immunoglobulin levels in the propositus and his brother

	Age, years	IgG mg/100 ml	IgA mg/100 ml	IgM mg/100 ml	IgD mg/100 ml	IgE ng/ml
Propositus	77	1,236	0	181	1 8	57 6
Brother	72	1,385	0	201	1 2	63
Normal levels in *our laboratory*		*800–1,600*	*140–400*	*50–200*	*0–40*	*150 510*

IgG, IgA, IgM, IgD single radial immunodiffusion method, IgE radioimmunoassay

G-200 gel filtration in 0 15 M Tris saline, pH 8 2, the first peak was pooled and concentrated to a protein content of 30–40 mg/ml, finally dialyzed against 0 15 M phosphate-buffered NaCl, pH 7 2

Red blood cell antibodies, leucocyte or platelet agglutination activity were investigated according to DACIE and LEWIS [9] Heterophilic antibody test was performed according to DAVIDSOHN and LEE [10]

Fluorescent antibodies against rat liver, stomach parietal cells and kidney, and *against thyroid were investigated utilizing the indirect immunofluorescence technique*, with fluorescein labelled rabbit serum anti human IgG, IgA, IgM (Behringwerke)

Results

Demonstration of selective IgA deficiency Using the immunodiffusion test, revealing protein concentrations as low as 0 01 mg/ml, no IgA could be detected in the patient's sera In addition to the propositus, his brother had a selective absence of IgA (table I) On the other hand, normal levels of immunoglobulin classes (IgG, IgA, IgM, IgD and IgE) were found in 10 relatives' sera Protein contents and electrophoretical patterns of the two sera were normal, as was the immunoelectrophoresis, except the absence of precipitin lines using anti-IgA sera

Characterization of anti-IgA antibodies Anti-IgA antibodies were detected by hemagglutination assay with different immunoglobulin coats (table II) Both sera reacted at a high titer against IgA from normal serum pool and all 7 IgA-myelomas (5 A_1, and 2 A_2), no other agglutinating antiglobulins were actually found Each agglutination system was also tested for inhibition by the autologous coat, other IgA proteins and pooled IgG or IgM All IgA samples inhibited this reaction at concentrations as low

Table II Agglutination of immunoglobulin-coated red blood cells

Immunoglobulin-coated to red blood cells purified from	Type of H-chains	L-chains	Serum samples propositus	brother
Pooled normal 110 sera	A	κ and λ	1 1 024	1 512
Myeloma (R O)	A₁	κ	1 512	1 512
Myeloma (C O)	A₁	κ	1 1 024	1 1 024
Myeloma (P U)	A₁	λ	1 512	1 1 024
Myeloma (C R)	A₁	λ	1 2,048	1 2,048
Myeloma (D E)	A₁	λ	1 1 024	1 512
Myeloma (A G)	A₂	κ	1 1 024	1 1 024
Myeloma (B L)	A₂	λ	1 1 024	1 1 024
Pooled normal 80 sera	G	κ and λ	0	0
Pooled normal 60 sera	M	κ and λ	0	0
Waldenström disease (D G)	M	κ	0	0
Waldenström disease (N E)	M	κ	0	0
Waldenström disease (R I)	M	κ	0	0

Table III Agglutination of IgA-coated red blood cells by IgG fractions of the propositus and his brothers sera

IgG fractions	IgA purified from myelomas R O	C R	P U	C O
Propositus	20 36[1]	1 27	10 18	5 09
Brother	62 7	0 48	31 35	1 9

[1] Minimal agglutining dose of IgG (mg 100 ml)

as 0 005 mg ml whereas pooled IgG or IgM did not In the double diffusion test, both sera failed to react against normal human serum and purified IgA at various dilutions The anti IgA activity was found only in the sera fractions containing IgG separated by gel filtration on Sephadex G 200 table III indicates the lowest IgG agglutinating concentration

Other antibodies studies Testing both sera for iso- and heterophilic antibodies (table IV) revealed that in the propositus serum there was a lower than normal titer of isoagglutinins and an absolute lack of heterophilic antibodies while his brother s serum was normal No autoantibod

Table IV Titer of isohemagglutinins and heterophilic antibodies

	Isohemoagglutinins			Heterophilic antibodies
	A_1	A_2	B	
Propositus	1 2	1 2	0	0
Brother	1 32	1 32	0	1 56

ies against rat liver, stomach parietal cells and kidney, and human thyroid were found by indirect immunofluorescence technique Precipitins against heterologous proteins were investigated by double diffusion test both sera failed to react against cow milk, animal sera (cow, horse, goat, sheep, rabbit, guinea pig), cow and horse macroglobulins

Discussion

That the anti-IgA antibodies can be responsible for transfusion reactions, has been previously described [3, 16, 24, 28, 29, 36, 44, 46] In fact, in our propositus we could exclude the presence of ABO incompatibility, hemolytic, leukocyte and platelets agglutinating, and anti-IgG or anti-IgM antibodies

The demonstration in our patient s serum of anti-IgA activity at a high titer against all IgA tested, which included that purified from the normal serum pool and from 7 IgA-myelomas (5 A_1 and 2 A_2), support the conclusion that the antibodies involved are class-specific, directed against the α-chains, produced by about 40% of subjects lacking IgA [2, 14] However, antibodies of allotypic specificity were also found in a patient lacking IgA [29], but they react against some IgA only, and at a weaker titer As shown by gel-filtration of the propositus' serum, anti-IgA antibodies belong to IgG class, according to other authors' results [24, 29, 36, 46], they are agglutinating only in fact, we could not detect any precipitation reaction in double diffusion tests

Because of the increased incidence of selective IgA deficiency in some families, we studied our propositus' relatives, his brother showed the same abnormality complete absence of IgA and anti-IgA antibodies, class-specific, belonging to IgG

Selective IgA absence has been reported in healthy subjects [7, 13, 18, 34] as well as associated with (a) atopic manifestations [1, 4, 22, 26, 41, 43] or various diseases [2, 5, 7, 8, 11, 19, 38, 48], (b) IgE deficiency [33], and (c) antibodies directed against auto- and heterologous antigens [2, 20, 23, 47] The only association in our cases was perhaps the presence of repeated mild respiratory infections in the propositus' brother.

Our findings regarding the low level of isoagglutinins and the absence of heteroagglutinins in the propositus are of some interest both these antibodies belong to IgM class, whose level was normal in his serum This fact might suggest an associated functional arrest in the development of IgM-committed B lymphocytes, this alteration would affect the ability to produce immunoglobulins of restricted heterogeneity, that is high affinity antibodies [40] Thus the defect in induction of B-cells terminal differentiation appears more severe in the propositus than in his brother

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Cytochemical Abnormalities of Atypical Erythroblasts in Acute Erythremic Myelosis[1]

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Key Words. Atypical erythroblasts · Cytochemistry · Di Guglielmo's disease · Erythremic myelosis · Esterase reaction

Abstract. Cytochemical properties of atypical erythroblasts and 'histioid' cells from the bone marrows of two patients with acute erythremic myelosis (Di Guglielmo's disease) were studied. In addition to strong PAS and strong nonspecific esterase positivity, intense specific esterase positivity was also detected in the majority of the erythroid precursors. Since specific esterase activity is considered to be a feature of granulocytic cells, its presence in erythroid precursors raises the possibility that the atypical erythroblasts of acute erythremic myelosis may share close metabolic relationships with granulocytic cells.

This report describes cytochemical abnormalities of atypical erythroblasts in two patients with acute erythremic myelosis (Di Guglielmo's disease). It is of particular interest that many of the erythroid precursors contained cytochemical evidence of granulocytic differentiation.

Materials and Methods

Bone marrow was obtained at the time of diagnosis from an 85-year-old Caucasian male and a 72-year-old Caucasian male with acute erythremic myelosis. The diagnosis of this unusual disorder was made according to accepted criteria [1, 7, 8, 11, 12, 15]. Films of bone marrow flecks were made between methanol-cleaned coverslips and stained with Wright's stain for conventional light microscopy. Other separate coverslips were stained for glycogen using the PAS (periodic acid Schiff) reagent [9], iron using the Prussian blue stain [3], specific and nonspecific esterase on the same coverslip [16], and amylophosphorylase [9].

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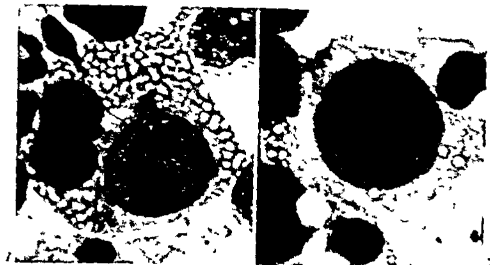


Fig. 1. Acute erythremic myelosis: giant, multinucleate erythroblast. ×830.
Fig. 2. Acute erythremic myelosis: large 'histioid' cell, perhaps a large reticulum cell, in the bone marrow. ×1,000.

Results

The bone marrows of these two patients exhibited typical features of acute erythremic myelosis [1, 7, 8, 11, 12, 15]. Most of the large aberrant erythroid precursors exhibited a megaloblastoid nuclear chromatin pattern and giant multinucleate erythroblasts were frequently observed (fig. 1). Unusual histiocytic-type cells were also seen (fig. 2) resembling those originally described by Di Guglielmo [7] as 'histioid' cells. Other cells appeared to combine features of both histiocytic and erythroid cells and could not be definitely classified as to type (fig. 3). Granulopoiesis was 'left-shifted', with increased numbers of progranulocytes. Myeloblasts constituted less than 5% of the marrow differential count, and none of them contained Auer rods.

The results of the cytochemical reactions are summarized in table I. The PAS stain demonstrated large block-like aggregates of glycogen within the cytoplasm of many of the erythroid precursors (fig. 4a). Diffuse PAS positivity was seen in the 'histioid' cells. Phosphorylase activity was intense in the majority of the erythroid precursors (fig. 4b), but could not be detected in the 'histioid' cells. Granulocytes and megakaryocytes showed strong phosphorylase activity.



Fig 3 Atypical cell exhibiting the nuclear chromatin pattern of proerythroblast and cytoplasmic features of a histiocyte or reticulum cell The origin and identity of this cell are difficult to classify × 1,200



Fig 4 *a* Strong PAS reaction in proerythroblast *b* Intense phosphorylase reaction in proerythroblast × 1 200

Table 1 Cytochemical abnormalities of atypical erythroid precursors in 2 cases of acute erythremic myelosis

Stain	Strength of reaction
PAS	4+
Prussian blue	2+
Phosphorylase	4+
Specific esterase	2 3+
Nonspecific esterase	3-4+

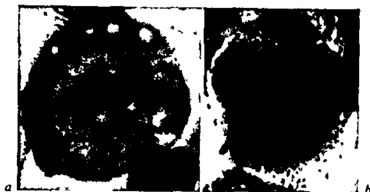


Fig 5 *a* Proerythroblast showing strong nonspecific esterase activity (dark gray cytoplasmic color) and punctate specific esterase activity *b* Large normoblast, showing intense specific and nonspecific esterase activity in the cytoplasm ×1,200

The Prussian blue stain demonstrated numerous ringed sideroblasts In some erythroblasts, a diffuse blue cytoplasmic color was seen In most of the atypical 'histioid' cells a similar faint diffuse blue coloration of the cytoplasm could be observed, and occasional discrete siderotic granules could be found

Intense nonspecific esterase activity was localized in a perinuclear zone in the proerythroblasts from the marrow of one of the patients, and intense diffuse nonspecific esterase activity was found in the majority of the erythroid precursors of the other patient (fig 5) Weak nonspecific esterase activity was detected in the 'histioid' cells of both patients Specific esterase activity was strongly positive in the mature granulocytes and granulocytic precursors Specific esterase activity could be identified in the majority of the erythroid precursors including those as early in development as the proerythroblast (fig 5a) and in most of the 'histioid' cells as well In some of the late erythroid precursors, specific esterase activity was intense (fig 5b) and in some cells occurred within vacuoles as well as in the cytoplasm

Discussion

Certain of the cytochemical findings in these two cases were in accord with those of others namely that the erythroid precursors of acute erythremic myelosis contain abundant amounts of glycogen demonstrable

by the PAS reagent [1, 9] ringed sideroblasts as in other forms of the Di Guglielmo syndrome including chronic erythremic myelosis and erythroleukemia [9, 12], and strong perinuclear nonspecific esterase activity [14] The strong phosphorylase activity in the erythroid cells of the present cases of acute erythremic myelosis has also been observed in erythroleukemia [9] and in chronic erythremic myelosis [13] and may constitute cytologic evidence for an abnormality in the glycogen biosynthetic pathway

Of particular interest in the present study was the finding of strong specific esterase activity in the majority of the erythroid precursors Specific esterase activity is generally associated with cells of granulocytic origin [16] and the specific esterase stain has been used as a 'marker' to identify these cells Occasional peroxidase positive granules have been noted in the erythroid precursors of patients with acute erythremic myelosis [2] and may have a significance similar to the finding of specific esterase activity in these cells namely that many of the erythroid precursors in acute erythremic myelosis demonstrate evidence of granulocytic differentiation In support of this viewpoint granules have been observed in the Golgi apparatus of atypical erythroblasts of acute erythremic myelosis by HUHN *et al* [10]

An erythroid cell demonstrating granulocytic properties would fit with the concept of DAMESHEK and BALDINI [4] and DAMESHEK [5, 6] who believed that the first stage of the Di Guglielmo syndrome was acute erythremic myelosis and the final stage was acute myeloblastic or myelomonocytic leukemia The present cytochemical studies would tend to support the viewpoint that there may be close metabolic relationships between granulocytes and erythroid precursors in acute erythremic myelosis and that these abnormal erythroid cells themselves may have a potential to differentiate along granulocytic lines Alternately it is conceivable that the finding of granulocytic properties in an erythroid cell in acute erythremic myelosis may be an epiphenomenon and bear no relationship to the ultimate development of acute myeloblastic or myelomonocytic leukemia that can occur in patients with this disorder [1, 4–6 12]

The significance of the strong nonspecific esterase activity in the erythroid precursors of acute erythremic myelosis is uncertain, since similar strong nonspecific esterase activity can be found in chronic erythremic myelosis and in pernicious anemia [14] Nonspecific esterase activity is usually associated with cells of monocytic or reticulum cell origin [16]

and the finding of nonspecific esterase activity in an erythroid precursor may indicate a close metabolic relationship between the reticulum cell and the erythroid precursor in some of these disorders [12].

As yet, the origin of the 'histioid' cell in acute erythremic myelosis has not been determined. The diffuse blue staining of the cytoplasm of these cells with the Prussian blue reagent suggests that they may share some of the properties of the erythroid precursors. Although these cells contain iron demonstrable by the Prussian blue reaction, it has not as yet been shown that hemoglobin synthesis is occurring within these 'histioid' cells and that they are, in fact, exhibiting a property normally attributed to erythroid precursors. The weak nonspecific esterase activity and weak specific esterase activity also suggest that these 'histioid' cells may be, in a sense, 'hybridized', and exhibit properties of reticulum cells and granulocytic cells within the same cell.

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Human Monocytopoiesis in Acute and Chronic Inflammation[1]

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Key Words Cell kinetics DNA synthesis Inflammation Monocytopoiesis Promonocytes

Abstract Monocytopoiesis was analyzed in patients with severe acute inflammations induced by surgical interventions as well as in others with mild chronic inflammations in connection with gastric or duodenal ulcers The state of acute inflammation was assumed to be associated with a high and steeply rising monocyte demand as opposed to the constant and relatively small monocyte recruitment in chronic inflammation In chronic mild inflammatory reactions DNA synthesis activity of promonocytes was increased by a factor of about two the promonocyte pool was normal In patients who underwent surgical operations changes in the following parameters were observed during the first 15 h after start of surgery (1) average increase in ^{3}H TDR labelling index by 38% (2) average enlargement of promonocyte pool by 34% (3) and relase of immature cells from the bone marrow into the blood Increase in DNA synthesis activity as well as expansion of the promonocyte pool cause an enhanced monocyte production rate The shift to the left in monocyte egress is equivalent to a reduced stem cell to blood transit time These variations permit short term adaptation of monocytopoiesis to varying demands

Macrophages predominate in the cellular infiltrates of inflammatory reactions [14] This statement is true except for the very early phase of the acute inflammation during which a short lasting wave of neutrophils may immigrate the site of reaction [11, 12] During acute inflammation macrophages are exclusively recruited from blood monocytes [10 15] During chronic inflammation the number of inflammatory macrophages at the reaction site is maintained by three different processes (1) mon

[1] Research supported by Kind Phillipp Donation

ocyte recruitment, (2) self-renewal of macrophages by mitosis, (3) selection of long living macrophage populations [14]. Thus, acute as well as chronic inflammation depend upon an adequate supply of blood monocytes. During chronic inflammation, the monocyte recruitment of inflammatory reactions may be constant as opposed to steeply rising as during acute inflammation.

The mobile reserve of functionally developed cells is small in monocytopoiesis and consists of the pools of circulating and marginating monocytes [3–7]. Therefore, adaptation of monocyte supply to a rapidly rising demand requires compensatory mechanisms. The present study seeks to investigate these mechanisms in patients with chronic inflammations and in others with acute traumatic inflammations induced by surgical operations.

Material and Methods

Subjects. Monocytopoiesis was examined in 4 patients aged 32, 39, 39 and 64 years. Two of them suffered from chronic gastric ulcers, the others from chronic duodenal ulcers. They underwent the following surgical procedures: pylorogastrectomy (2 cases), pylorectomy and vagotomy combined with pyloroplastic surgery.

Blood monocytes were examined in 4 additional hematologically normal patients undergoing the following operations: pylorogastrectomy, cholecystectomy, mammaryplastic surgery and bilateral subcutaneous amputation of the breast due to mastopathia chronica cystica.

All patients gave their informed consent to the study.

Analysis of monocytopoiesis. Two bone marrow samples were obtained by sternal puncture in each of the 4 cases examined. The first sample was collected during the first minutes after onset of general anesthesia. The following samples were collected 13, 14.5, 14.5 and 17 h thereafter, respectively. The marrow samples were immediately anticoagulated with 0.5% Na_2EDTA in 0.7% NaCl, filtered through nylon gauze and carefully rinsed free from contaminating blood with 2 ml of autologous serum. Subsequently the particles were incubated for 1 h at 37 °C together with 2 ml of autologous serum containing 12 μCi ^3H thymidine (^3H TDR).

Smears were prepared from the marrow particles. Some of them were stained by May Grünwald Giemsa stain. In these preparations 1,000 nucleated cells were scanned to evaluate the marrow differential count. On other smears, promonocytes were identified by simultaneous detection of NaF resistent and NaF sensitive naphthol AS D acetate esterases [13]. The latter smears were used for dipping film autoradiography (Ilford L_4 emulsion). After 14 days of exposure and photographic processing, nuclei were stained through the film layer with Mayer's haematoxilin. In addition to band and segmented neutrophils, 3,000 other nucleated marrow cells were scanned to determine the relative number of promonocytes in the myelogram. To determine the ^3H TDR labelling index (LI), 1,000 promonocytes were counted. Nuclei overlaid by more than 4 grains were considered as labelled. In addition,

these promonocytes were classified into 4 groups according to morphological criteria (see below).

Calculation of the medullar promonocyte pool was based on data of total marrow cellularity determined by Donohue *et al.* [1]. By excluding band forms and segmented neutrophils from the calculations, modification of the results by the postoperative mobilization of the medullary neutrophil reserve was avoided.

Study of blood monocytes. Venous blood samples collected repeatedly before and after the surgical intervention served to determine the behaviour of blood monocyte counts, of monocyte morphology, and of monocyte ^{3}H-TDR LI.

Blood leucocyte counts were obtained with a Coulter Counter. A number of leukocytes with 20 monocytes were scanned on May-Grünwald-Giemsa stained blood smears to establish differential blood counts. The monocyte blood count was calculated from both results.

Samples of 4 ml of venous blood were mixed with Na_2EDTA-plasmagel (prepared by dissolving 1.107 g Na_2EDTA and 1.4 g NaCl in 100 ml distilled water and adding 100 ml plasmagel) and 8 μCi ^{3}H-TDR (specific activity 5 Ci/mM). The aliquots were then allowed to sediment for 20 min at 37 °C. The supernatant was spun for 5 min at 150 g and smears were prepared from the sediment. The monocytes in these smears were identified by the combination of NaF-resistent and NaF-sensitive naphthol-AS-D-acetate esterase as mentioned above. Subsequently the smears were processed by autoradiography using Ilford L_4 emulsion and 2 weeks' exposure. Thereafter, nuclei were stained with Mayer's haematoxilin through the film layer. 1,000 monocytes were microscopically classified into 3 groups according to morphological criteria (see below) and according to the uptake of ^{3}H-TDR. Cells overlaid by more than 4 grains were considered as labelled.

Morphological classification of monocytes and promonocytes. Promonocytes were classified into 4 groups according to nucleus morphology: type I promonocytes of lymphocyte size displayed small round or oval nuclei with tight chromatin, type II promonocytes displayed large round or oval nuclei of approximately myelocyte size, type III promonocytes had large and slightly folded nuclei, and type IV promonocytes had distinctly folded nuclei.

The morphological features of type II–IV promonocytes also were used to classify blood monocytes into 3 groups: monocytes with large round or oval nuclei, monocytes with slightly folded nuclei, and monocytes with distinctly folded nuclei. Monocytes with small round or oval nuclei occurred only occasionally in the peripheral blood.

Results

Monocytopoiesis. In table I the percentage of promonocytes in the myelogram of patients with gastric or duodenal ulcers is compared with that of 10 healthy individuals [8]. Before surgery, the relative number of promonocytes only slightly exceeded the normal range. During the 13- to 17-hour intervals following initiation of gastric surgery, the fraction of promonocytes rose by a mean of 37%.

Table I. Postoperative changes in the fraction of medullar promonocytes

Diagnosis	Surgery	Percentage of promonocytes¹			
		pre-operative	post-operative %	interval, h	normal
Gastric ulcer	pylorogastrectomy	5.1	6.4	17	–
Gastric ulcer	pylorectomy	4.2	6.2	14.5	–
Duodenal ulcer	vagotomy and pyloroplastic surgery	4.4	5.7	14.5	–
Duodenal ulcer	pylorectomy	4.9	6.8	13	
Mean ± SD		4.6 ± 0.4	6.2 ± 0.5		4.0 ± 0.7

The means and standard deviations of the results are compared with results of 10 healthy individuals [8].

¹ The relative number of promonocytes refers to all nucleated bone marrow cells apart from band forms and segmented neutrophils which were omitted in order to avoid the data of promonocytes to be influenced by mobilization of the neutrophil reserve.

The kinetic parameters of monocytopoiesis are listed in table II. As there were no significant differences in individual patients, the results were considered as samples of one population and only averages and standard deviations are reported. Before surgery, the total promonocyte pool of the bone marrow in the 4 patients with peptic ulcers was normal. DNA synthesis activity, as judged by the percentage of labelled promonocytes (LI) following incubation with ³H-TDR exceeded, however, normal level by a factor of about two. The increase of ³H-TDR LI *uniformly* comprised all types of promonocytes. Postoperative reexamination revealed a remarkable increase in the two parameters determined, the mean *increments being 34% for the total medullar promonocyte pool* and 38% for the ³H-TDR LI of pooled promonocytes. Type I promonocytes showed the most pronounced changes. They were less in type II promonocytes. Only little variation occurred in type III and type IV promonocytes.

Blood monocytes. Postoperative behaviour of blood monocytes in the 4 patients examined was similar (fig. 1). The blood monocyte counts behaved irregularly without a clear-cut decrease as suggested by the rising monocyte influx into the site of the inflammatory reaction. About 15–25 h after beginning of surgery the fraction of monocytes with large

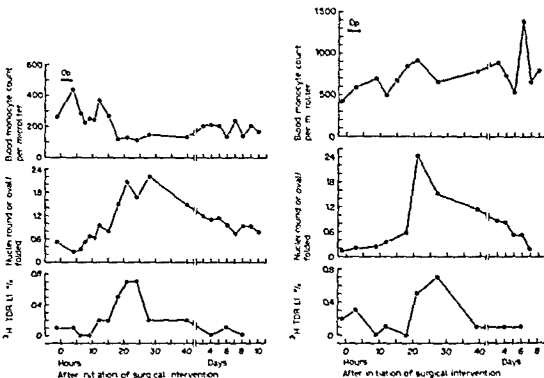
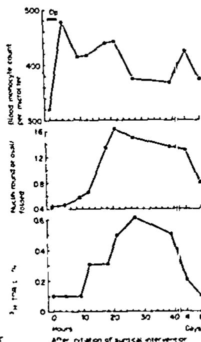
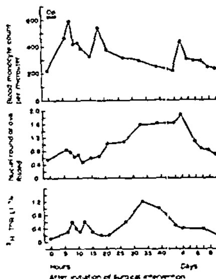


Fig. 1. Postoperative behaviour of blood monocytes in 4 patients undergoing cholecystectomy (a), partial gastrectomy (b), mammaryplastic surgery (c) or bilateral subcutaneous amputation of the breast (d).

round or oval nuclei increased markedly at the expense of monocytes with folded nuclei. Later on, the fractions of these two cell types gradually returned to base line. The variations in frequency distribution of the different types of promonocytes were paralleled by a nearly simultaneous rise and decrease of monocyte ³H-TDR LI.

It has been shown in previous studies [9] that monocytes with round or oval nuclei demonstrate the highest ³H-TDR LI among the different types of blood monocytes. They represent the most immature monocyte fraction occurring in the blood. In contrast to this, monocytes with distinctly folded nuclei demonstrate the lowest ³H-TDR LI and represent the most mature form of blood monocytes [9]. Therefore, the simultaneous rise of both parameters, i.e. of the fraction of monocytes with round or oval nuclei and of ³H-TDR LI indicates a shift of monocyte marrow release in favour of immature monocytes.





Discussion

In patients with chronic inflammations due to gastric or duodenal ulcers the medullar promonocyte pool was normal. DNA synthesis activity exceeded the normal mean value by a factor of about two (table II). This observation demonstrates that proliferative activity of promonocytes may be stimulated even by relatively mild inflammatory reactions.

The rise in DNA synthesis activity observed means that proliferative activity in normal monocytopoiesis is not fully utilized. Under normal conditions only about 36% of the promonocytes proliferate [5] as calculated from mean cell cycle time of 30 h [7], mean DNA synthesis time of 10 h, and mean ^{3}H-TDR LI of 12% [8]. Increase in proliferation activity may result from triggering G_0 phase cells into the cell cycle as well as by shortening cell cycle time primarily at the expense of the G_1 phase. The latter mechanism has been proved by demonstrating shortened cell cycle

ble II Monocytopoiesis in patients suffering from gastric or duodenal ulcers before and 13-17 gastric operations

onocyte ecursors		Operation before	after	p[1]	Normal	Mean ratio normal befo
pe I	pool	42 9±15 2	126 1±33 1	<0 0025	30 1±11 9	1/1 4/4 2
	LI	11 5±3 8	23 6±7 5	< 0 05	7 1±5 5	1/1 6/3 3
pe II	pool	171 4±11 2	352 5±37 5	=0 0025	176 5±26 9	1/1/2 0
	LI	16 9±6 2	31 6±7 3	<0 0025	9 7±2 2	1/1 7/3 3
pe III	pool	307 0±20 5	238 0±33 7	< 0 025	296 9±66 2	1/1/0 8
	LI	21 7±5 2	34 5±8 6	< 0 01	10 1±1 8	1/2 2/3 4
pe IV	*pool*	*139 5±46 4*	*167 8±32 1*	*n s*	79 3±26 7	*1/1 6/2 1*
	LI	38 7±7 4	36 6±4 0	n s	24 9±0 25	1/1 6 1 5
otal	pool	660 0±61 2	885 5±65 0	<0 0025	584 4±117 6	1/1 1/1 5
	LI	*23 4±4 4*	*32 2±6 6*	*< 0 025*	*12 0±1 8*	*1/1 9 2 7*

Data express mean and standard deviation of 4 patients The results evaluated in patients are con *th data obtained from normal individuals [8]*

Pools are expressed as promonocytes × 10^8/kg body weight, LI = ^{3}H-TDR labelling index *i* pressed percentages

[1] Probability level that sizes of promonocytes before operation equal those of promonocyte eration t test of dependent data

times in a septicaemia patient [8], and during acute inflammation in mice [2], and in rats [16]

In acute inflammation as induced by surgical operations changes in three parameters were observed (1) expansion of the promonocyte pool, (2) increase in DNA synthesis activity of promonocytes exceeding that of chronic inflammation, and (3) release of immature monocytes from the bone marrow into the blood These observations agree with findings in animal studies [2, 16]

It is interesting to note that the rise in both components, i e in pool size and DNA synthesis activity, was particularly pronounced for type I promonocytes This indicates that, among the morphologically different precursors, small lymphocyte-like promonocytes are equipped with the highest proliferation reserve

The increase in *DNA synthesis activity of promonocytes as well as* expansion of the promonocyte pool cause a rise in monocyte production rate In addition the concomitant 'shift to the left' in monocyte marrow

egress is equivalent to a reduced mean stem-cell to-blood transit time These mechanisms permit short term adaptation of monocytopoiesis to varying demands

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Treatment of Acute Lymphocytic Leukemia in Uganda

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Key Words. Acute lymphocytic leukemia · Leukemia treatment · Uganda

Abstract. The hematologic remission rate of 17 black Ugandan children with acute lymphocytic leukemia treated with prednisone and vincristine was 41%. There was no correlation of the remission rate with any clinical or laboratory finding at the time of diagnosis. The frequency of remission approximated that of similarly treated black American children but was significantly less than that achieved in white children. Whether this poor response to therapy as compared to the response of white children represents genetic, environmental or socioeconomic factors is discussed.

The hematologic remission rate and length of survival in black American children with acute lymphocytic leukemia was found by WALTERS *et al.* [10] to be significantly less than in white American children. Our experience with induction of hematologic remissions in 17 African children with the same disorder is described in this report.

Patients and Methods

23 black Ugandan children, age 16 years or less, were diagnosed at Mulago Hospital, Kampala, as having acute leukemia between November 1971 and August 1973. 17 had acute lymphocytic leukemia (ALL), and of these 3 were female. The mean age of the 17 was 6.3, the median 5, with a range of 0.5–15 years.

The diagnosis was made by cytological examination of the blood and bone marrow [7] using Leishman's and Giemsa's stain, respectively. When the cytological diagnosis was uncertain, a peroxidase and periodic acid-Schiff stain were performed and the criteria of HAYHOE *et al.* [7] were used in interpretation. A hema-

tologic remission was defined as a cellular marrow demonstrating adequate hematopoiesis and containing less than 5% lymphoblasts with less than 20% of cells being of the lymphocytic series [3] The patient also had to be free from symptoms and physical findings attributable to leukemia

The patients were treated for induction of remission for 4–6 weeks as in patients Marrow aspiration was performed weekly if there were no lymphoblasts in the peripheral blood The drug regimen for remission induction was vincristine 1.5–2 mg/m² intravenously weekly for 4–6 weeks, and prednisone 40–60 mg/m² orally, daily for 4–6 weeks Allopurinol was also given during the treatment period

Results

Of the 17 children with ALL, 7 or 41% had hematological remission Three patients died before the second week of treatment, excluding these 3 the remission rate increased to 50% Of the 18 black and 139 white American children treated with the same regimen by WALTERS *et al* [10] the remission rate was 61 and 97%, respectively The hematologic remission rate of 41% in the Ugandan children was significantly lower than in white ($p<0.001$) but not black American children

There was no statistically valid correlation between age, distance from home to the hospital initial hemoglobin level, white cell count, spleen size and degree of lymph node enlargement and the remission rate Distance of a hospital from a patient's home is stressed because rural areas have fewer medical facilities, making delay from onset of symptoms to initiation of treatment likely The hemoglobin level, leukocyte count and size of the lymph nodes and spleen were used to indicate the extent of disease at the time of diagnosis

The male female ratio and median age of Ugandan children was 4.6 1 and 5 years The figures for black Americans were 1.3 1 and 6.6 years and for white Americans 1.7 1 and 4.8 years [10] The reasons for the increased male female ratio in Ugandans is not apparent Of the 23 children with acute leukemia in Uganda, 26% had acute granulocytic leukemia Only 19% of white American children had acute granulocytic leukemia, but a greater fraction (32%) of black Americans had this disorder [10]

Discussion

The hematologic remission rate for white children with ALL is approximately 90% [8, 10] The rate for black Americans is 61% [10] and

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There was no statistically valid correlation between age, distance from home to the hospital, initial hemoglobin level, white cell count, spleen size and degree of lymph node enlargement and the remission rate. Distance of a hospital from a patient's home is stressed because rural areas have fewer medical facilities, making delay from onset of symptoms to initiation of treatment likely. The hemoglobin level, leukocyte count, and size of the lymph nodes and spleen were used to indicate the extent of disease at the time of diagnosis.

The male female ratio and median age of Ugandan children was 4.6:1 and 5 years. The figures for black Americans were 1.3:1 and 6.6 years and for white Americans 1.7:1 and 4.8 years [10]. The reasons for the increased male female ratio in Ugandans is not apparent. Of the 23 children with acute leukemia in Uganda, 26% had acute granulocytic leukemia. Only 19% of white American children had acute granulocytic leukemia, but a greater fraction (32%) of black Americans had this disorder [10].

Discussion

The hematologic remission rate for white children with ALL is approximately 90% [8, 10]. The rate for black Americans is 61% [10] and

for black Ugandans 41%, as found in the present study The three groups were treated similarly The reason for such differences in the response of children of both races is unkown In our patients there was no correlation between remission rate and any finding at the time of diagnosis Others have found the frequency of remission to vary inversely with age but not to be affected by blood counts or duration of symptoms at the time of diagnosis [6] A study in which prednisone alone was used as therapy showed a leukocyte count greater than 50,000 to be associated with lower remission rates [11] WALTERS *et al* [10] found no correlation between remission rate and any finding at the time of diagnosis in their study of black and white American children

An intriguing question is if genetic differences between races can account for differences in response to therapy in ALL In favor of genetic factors being active are the findings in black American and Ugandan children of a low incidence of ALL [2, 4], an absence of the childhood peak in incidence [2, 10], and an increased proportion of acute granulocytic leukemia when compared to white children [10] There is evidence, though, that the paucity of ALL in Ugandans is on the basis of underdiagnosis rather than a true deficit of disease [2, 9]

Against genetic factors is the observation that most black American children with ALL came from low income families, were poorly nourished and had more advanced disease (delayed diagnosis) at the time of diagnosis than white children It was suggested that these socioeconomic factors may have been partially responsible for the poorer response in black children [10] In Uganda the influence of environment on certain malignancies is important The incidence of carcinoma of the liver and Burkitt's lymphoma varies in different regions of the country and can be correlated with the presence of an agent in the environment [1, 5] However, in Uganda there is no evidence that this occurs with leukemia [2] At present, the decreased incidence and low frequency of hematologic remissions in black children with ALL when compared to white children remains unexplained

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Thrombopoietin Activity in Mice Following Immune-Induced Thrombocytopenia

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Key Words. Megakaryocyte kinetics · Radiobioassay · Thrombocytopenia · Thrombopoietin

Abstract. Thrombopoietin levels in thrombocytopenic mice assayed by ^{75}Se-selenomethionine incorporation into blood platelets reached a maximum 12 h after the induction of an acute immune thrombocytopenia that was more than twice the value in control mice. The implications of this finding are discussed with reference to the kinetics of megakaryocyte production.

The response of megakaryocytes to an immune induced thrombocytopenia may be mediated by a humoral thrombocytopoietic activity (thrombopoietin) [1, 2, 7]. The temporal relationship of this response has been assessed by following the change in a presumed class of megakaryocyte precursors, the small acetylcholinesterase positive cell [6]. It may be reasonably assumed that the changes observed reflect changes in the level of thrombopoietin.

The study reported here approaches this question directly by utilizing a ^{75}Se-selenomethionine bioassay for plasma thrombopoietin activity in the plasma of mice as a function of time after the induction of an acute immune thrombocytopenia.

Material and Methods

Animals. Male Swiss mice (25–35 g) maintained in the Radiobiological Institute were used both as thrombopoietin donors and recipients, and New Zealand white rabbits (7 kg) were used to prepare rabbit anti mouse platelet serum (RAMPS).

Preparation of RAMPS. On day 0, 2×10^9 mouse blood platelets were injected into rabbits via the ear vein with a second injection of platelets given on day 8 and the rabbits exsanguinated on day 18. The serum was collected, heat inactivat-

ed (56 C for 45 min) and absorbed twice (37 C for 30 min) with equal amounts (v/v) of packed cells from defibrinated mouse blood

Preparation of plasma for thrombopoietin assay Mice received RAMPS intravenously (0 4 ml/kg) and at various times thereafter were exsanguinated and their blood collected in heparin (10 U/ml) and pooled The plasma collected after centrifugation was filtered (0 45 μm) and stored at -20 °C for no longer than 1 week

Thrombopoietin bioassay Groups of 5 recipient mice received the test plasma (0.3 ml mouse via the tail vein) administered as two separate injections 1 day apart One day after the second injection each recipient mouse received 2 μCi of [75]Se selenomethionine (Amersham 1–6 mCi/mg) in saline Two days later these mice were injected i p with 75 U heparin and exsanguinated into 2% EDTA K_2 The blood was diluted 1 10 with ACD and centrifuged over liquid silicone (13 400 g for 5 min) in 50 μl volumetric capillary tubes [8] Platelets at the platelet plasma interface were pooled their number counted electronically and a sample counted for γ-emission in a Nuclear Chicago sodium iodide well type gamma counter The incorporation of [75]*Se* selenomethionine was then expressed as cpm 10^8 platelets and this value used as an estimate of the thrombopoietin activity of the test serum

Results

Plasma thrombopoietin activity from thrombocytopenic mice is presented in figure 1 as a function of time after the injection of RAMPS During the first 6 h after mice were injected with either RAMPS or normal rabbit serum plasma thrombopoietin activities of both groups were similar 12 h after treatment however maximum thrombopoietin activity was measured in the plasma of acutely thrombocytopenic mice and was more than twofold higher than that found in the plasma of the control mice Increased levels of thrombopoietin activity persisted for up to 48 h after treatment The control group stayed within the initial control range throughout the 48 hour period

Platelet counts of the mice given RAMPS were less than 5‰ of normal within 15 min after its administration and remained at this value throughout the subsequent 48 h Platelet counts for the control mice which received normal rabbit serum and the two recipient groups which received either thrombopoietin or control plasma remained within normal limits throughout the test period (1 1 × 10^9 platelets/ml whole blood + 0 2 [1 SD])

Discussion

Although increased use has been made of radioisotopic bioassays for measuring thrombopoietin activity utilizing both [75]*Se selenomethionine*

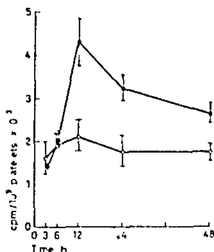


Fig. 1. The incorporation of ⁷⁵Se-selenomethionine into the blood platelets of mice receiving plasma obtained from mice which had previously received RAMPS (solid symbols). The abscissa indicates the time at which the plasma was collected from the RAMPS-treated mice. The control represents the effect of plasma obtained from mice which had received serum from normal rabbits (open symbols). Errors shown represent ± 1 SEM.

incorporation [5, 12, 13] and ³⁵S-sulfate incorporation [2, 3] into newly produced blood platelets, no information is available concerning the manner in which levels of thrombopoietin activity change in response to an increased demand for platelets. This information is important if one assumes that the accelerated megakaryocytosis reported by JACKSON [6] could be the result of specific stimulation by thrombopoietin induced by the thrombocytopenia.

The finding of maximum plasma thrombopoietin activity 12 h after an acute thrombocytopenia is compatible with the proposed role of thrombopoietin as a humoral regulator of thrombocytopoiesis [1, 2, 4, 7]. If one assumes that thrombopoietin accelerates thombocytopoiesis by acting at the level of the morphologically unidentified megakaryocyte precursor cell compartment (pluripotential or polyploid) and that the cell generation time in this compartment is approximately the same as that for the proliferating megakaryocyte compartment (9.5 h) [9], then one might expect to observe increase levels of thrombopoietin preceding a 'reactive' megakaryocytosis. Such a megakaryocytosis occurs within 24–72 h after the induction of thrombocytopenia [4, 7, 10].

Recently, the kinetics of the megakaryocytosis following acute platelet depletion have been measured by utilizing the ^{3}H thymidine-labeling index of newly synthesized megakaryocytes [11] and a quantitative cytochemical assay for megakaryocyte precursors [6] The study by JACKSON [6] has shown that the maximum increase in the number of megakaryocyte precursors may occur as early as 6–9 h after an acute, immune-induced thrombocytopenia, while ODELL *et al* [11] have shown that the increase in the mitotic index of megakaryocytes is observed as early as 12 h and reaches a maximum by 30–36 h after the thrombocytopenia

Our finding of increased thrombopoietin activity commencing between 6 and 12 h after an acute thrombocytopenia which reaches a maximum at 12 h fits within the framework of the above studies and is consistent with the concept that thrombopoietin is a humoral factor responsible for accelerating megakaryocytopoiesis in response to thrombocytopenia

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A New Family with Congenital Factor XII Deficiency

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Key Words Blood coagulation Factor XII deficiency Hageman trait Partial thromboplastin time

Abstract The case of a patient with Hageman trait and his family study are reported Commercial plasma thromboplastin time (PTT) reagents showed a good sensitivity for detecting the plasma defect By prolonging the incubation time of the mixture containing PTT reagent and factor XII-deficient plasma the abnormal coagulation times were not corrected Thus a concomitant Fletcher factor deficiency could be excluded

Hageman trait is a rare coagulation abnormality Since the initial description by Ratnoff and Colopy in 1955 [7], 115 cases have been reported until 1968 [8] since then 16 more have been published [2] The first case of Hageman trait in Italy was described by Girolami *et al* [5] in 1967 and since then we have only knowledge of another case [6]

The object of the present paper is to present an Italian family with congenital factor XII deficiency and to investigate the sensitivity of three commercial partial thromboplastins to the defect in factor XII plasma

Material and Methods

Blood was collected in plastic syringes and added to polycarbonate centrifuge tubes containing sodium-citrate anticoagulant 3.8% (1 ml anticoagulant 9 ml blood) The samples were centrifuged for 20 min at 3 000 rpm and plasma was re

[1] We wish to express our gratitude to Dr Girolami for the factor XII assay performed in our propositus

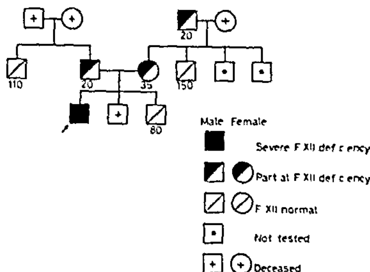


Fig. 1. Family pedigree. The numbers indicate the values of factor XII concentration. The arrow indicates the propositus.

moved using plastic droppers. The plasma was aliquoted into plastic tubes and was either tested immediately or quick frozen and stored at −20 °C until tested. Coagulation screening tests and coagulation factor assays were done by methods previously reported from this laboratory [3].

The factor XII assays were performed by a one stage plasma thromboplastin time (PTT) method using as substrate both a commercial deficient plasma (from Dade) and a congenitally deficient plasma factor XII (less than 1%) from the patient of GIROLAMI *et al.* [5]. Comparative PTT studies were done with the reagents purchased from Stago (cephalin+kaolin), Dade (cephalin+ellagic acid), Warner Chilcott (cephalin+celite).

Case Report

The propositus was a 8 year-old child. At 5 years he underwent tonsillectomy without excessive bleeding. The present coagulation screening tests were done because he had to undergo inguinal herniectomy. No blood transfusion was given during the operation and the patient did not bleed abnormally. The patient's parents came from the same country but were not related. No history of bleeding or thromboembolic accidents could be recorded in the family members.

Figure 1 gives the family pedigree: the propositus should be considered as homozygote while the parents and the maternal grandfather are heterozygotes.

Results

The results of haemostatic tests are reported in table I. No significant difference was noted between glass clotting time and plastic clotting time

Table I Coagulation study in the propositus

	Propositus	Normal values
Bleeding time (Ivy), min	5	3-9
Platelet count, $\times 10^3/\mu l$	250	200-400
Platelet adhesiveness (Salzmann), %	28	20-40
Platelet aggregation ('threshold concentration')		
ADP, $\times 10^{-6}$ M	0 5	0 2-1 3
Adrenaline, $\times 10^{-6}$ M	0 3	0 08-0 5
Clot retraction, %	90	80 100
Glass clotting time, min	20	5-10
Plastic clotting time, min	22	15-30
PTT, Howell time	see table II	
Prothrombin time, sec	13	13-14
Fibrinogen, mg%	400	200-400
Factor VIII, %	65	50 150
Factor IX, %	120	50 150
Factor XI, %	180	50 150
Factor XII, %		
Commercial substrate plasma	1 8	50-150
Congenitally deficient plasma	1 5	

The values of factor XII refer to the average of three determinations carried out on different occasions

Table II Screening tests on factor XII-deficient plasma

	Propositus	Normal values
Howell time, sec	216	129
PTT + activator[1]		
Cephalin + Kaolin, sec	55	45
Cephalin + ellagic acid, sec	75	36
Cephalin + celite, sec	140	58

[1] The reported PTT have been obtained at the recommended incubation time for each reagent

The plasma concentrations of factor XII were about 1.5-1 8%, respectively, using congenitally factor XII-deficient plasma and commercial factor XII-deficient plasma

The screening tests on the propositus plasma are reported in table

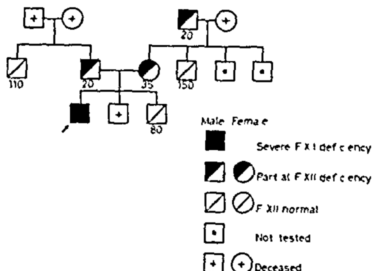


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Results

The results of haemostatic tests are reported in table I No significant difference was noted between glass clotting time and plastic clotting time

increasing incubation time may account for a Fletcher factor deficiency. The abnormal coagulation times of our patient were not corrected by prolonging the incubation time of PTT reagents. Therefore this property is unique of Fletcher-deficient plasma and gives further evidence of the separation of Fletcher factor from factor XII.

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Table III Sensitivity of various PTT reagents to the factor XII-deficient plasma at different incubation times

Incubation time min	Cephalin + kaolin	Cephalin + celite	Cephalin + ellagic acid
2	64	136	75[1]
3	58[1]	138	72
4	63	133	69
5	54	140[1]	65
7	62	147	63
10	63	146	71
Normal pool[2]	45	58	36

[1] *Result at recommended incubation time for each reagent All tests were done using Clot Timer apparatus, and are reported as the mean (sec) of two determinations*

[2] Normal values for the incubation time recommended for each reagent

II All the tests using calcium, or cephalin plus activators showed prolonged clotting times

PTT were also performed at different incubation times The results are presented in table III Prolongation of the incubation time to 10 min did not cause a significant variation of clotting times with all reagents tested

Discussion

Patients with congenital deficiency of factor XII have little or no tendency to spontaneous haemorrhages [4] and surprisingly, may have thromboembolic accidents [2] including thrombophlebitis, pulmonary embolism and particularly myocardial infarction [9] No thromboembolic attacks have been noted in members of this family The homozygote patient here presented underwent two operations (tonsillectomy and herniectomy) and no abnormal bleeding was noted during or after the surgical procedures Never was he given blood or plasma transfusions Platelet adhesiveness *in vitro* and platelet aggregation induced by ADP and adrenaline were normal

All commercial PTT reagents showed a significant prolongation of the clotting time at the recommended incubation times According to ABILGAARD and HARRISON [1] the correction of the prolonged PTT by

increasing incubation time may account for a Fletcher factor deficiency The abnormal coagulation times of our patient were not corrected by prolonging the incubation time of PTT reagents Therefore this property is unique of Fletcher-deficient plasma and gives further evidence of the separation of Fletcher factor from factor XII

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Absence of the Y Chromosome in Patients with Chronic Granulocytic Leukaemia

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Key Words Chronic granulocytic leukaemia Karyotype Y chromosome

Abstract The Y chromosome was missing from the cells of aspirated bone marrow in 2 patients with chronic granulocytic leukaemia (CGL) In both cases blast cell transformation occurred within 17 months of presentation Their short course is in contrast to the hypothesis that absence of the Y chromosome in CGL is compatible with long survival

Absence of one of the small acrocentric chromosomes of the G group has been described in several male patients with chronic granulocytic leukaemia [1, 2, 5, 6, 8, 9, 12–15, 17–20, 22], and there is now agreement that these patients lack the Y chromosome Nearly all patients with this unusual karyotype have had a benign variety of chronic granulocytic leukaemia (CGL) and, although their good prognosis has recently been questioned [13] it is accepted that this chromosomal abnormality is associated with long survival We describe here two patients with CGL in association with an absent Y chromosome and a rapid progression to the blast cell phase of the disease

Methods

Estimations of haemoglobin concentration and total WBC were performed using a Coulter S electronic blood cell counter The differential WBC platelet counts leucocyte alkaline phosphatase (LAP) score and examination of blood and bone marrow films were performed by standard haematological methods [4]

Cytogenetic studies on skin blood and bone marrow aspirates were performed by a modification of the method of WOODLIFF [23] Approximately 0.5 ml of bone

marrow is aspirated into a mixture of 16 ml tissue culture medium (TC 199) and 4 ml fetal bovine serum containing 0.5 ml heparin and 0.3 ml of colcemid After incubation for 2 h at 37 C, the cells are harvested by centrifugation resuspended in 0.075 M potassium chloride and fixed in a methanol acetic acid mixture using several changes of fixative The fixed cells are centrifuged stored at −20 °C over night resuspended in fresh fixative and stained with aceto orcein before microscopic examination and photography

Fluorescent staining was carried out using a modification of a previously described method which has been shown to give a more exact identification of the Y chromosome [16]

Case Reports

Case 1 A healthy 64-year-old phenotypically normal male the father of 2 children had a WBC of 40 000 /μl on routine testing (polymorphs 68%, lymphocytes 2%, monocytes 2%, basophils 7%, myelocytes 18% and myeloblasts 3%) His spleen was palpable 6 cm below the left costal margin but no other abnormal physical signs were found Haemoglobin concentration was 14.5 g dl platelet count 174 000 /μl and LAP score 5 (normal 15–100) Marrow aspiration showed marked myeloid hyperplasia

Treatment was started with busulphan 6 mg daily reducing to 1 mg daily in November 1972 when control of the disease occurred In January 1974 the platelet count fell to 9 000 /μl and he deteriorated with spontaneous skin bruising mucous membrane bleeding and a falling haemoglobin concentration The WBC rose to 66 000 /μl (20% myeloblasts) and a bone marrow aspirate contained 40% myeloblasts Following treatment with a combination of hydroxyurea, dexamethasone and 6-mercaptopurine his WBC fell transiently to 9 000 /μl (4% myeloblasts) but he again deteriorated when this treatment was discontinued A further marrow aspirate (March 1974) contained 59% blast cells and did not show any megakaryocytes. He died secondary to gastro-intestinal blood loss in August 1974

Case 2 A 61 year-old phenotypically normal male presented in December 1971 with a 3 month history of breathlessness on exertion He had been married for 40 years but had no children Examination was normal apart from splenomegaly 13 cm below the left costal margin and hepatomegaly 15 cm below the right costal margin His blood showed haemoglobin concentration 11.3 g dl WBC 124 000 /μl (polymorphs 51%, lymphocytes 2%, eosinophils 2%, myelocytes 27%, promyelocytes 11%, myeloblasts 7%) platelet count 154 000 /μl LAP score 5 (normal 15–100) Bone marrow aspiration showed an increase in all myeloid elements consistent with a myeloproliferative process Busulphan 6 mg daily resulted in clinical remission of his disease and a return of his blood picture to normal The busulphan was reduced to a maintenance dose of 1.5 mg daily in January 1972, and his progress was uneventful for 15 months thereafter In May 1973 he noticed tender swellings in both sides of his neck and examination showed multiple mobile lymph nodes in both supraclavicular fossae axillae and groins His spleen was palpable 5 cm below the right costal margin and there were several raised red lumps approximately 0.5 cm in diameter around the left nipple Biopsy of a cervi

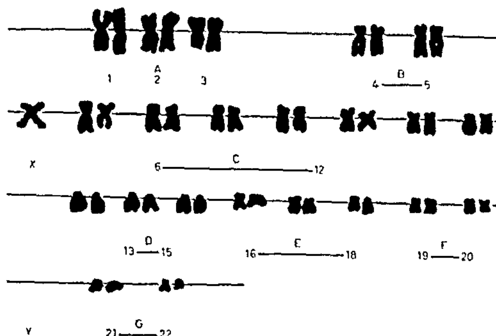


Fig 1 Chromosomal karyotype from a bone marrow cell (case 1)

cal lymph node and of a skin deposit showed infiltration with primitive granulocytes His blood showed haemoglobin concentration 8 8 g/dl, WBC 19 800/µl (polymorphs 54%, lymphocytes 9% monocytes 2%, eosinophils 1% basophils 2% myelocytes 20% promyelocytes 4%, myeloblasts 8%) platelet count 96 000/µl Red cell morphology was normal Bone marrow aspirate was hypocellular, the bone marrow trephine biopsy showed only a slight increase in reticulin

He deteriorated with increasing lymphadenopathy, a persistently low haemoglobin concentration, increasing blast cell count and increasing transfusion requirements His marrow remained hypocellular and his disease was resistant to further chemotherapy, including dibromanitol, 6-mercaptopurine and hydroxyurea He died in December 1973, but permission for a post mortem examination was refused

Chromosome Analysis

In case 1, the bone marrow cells at presentation showed a 2n 45 XO $22q^-$ karyotype (fig 1) The chromosomes from the G group are shown in figure 2 Further studies in the blast cell phase (table I) showed multiple abnormalities, the majority of cells again had a 2n 45 XO 22q karyotype, but several aneuploid cell lines contained the Y chromosome

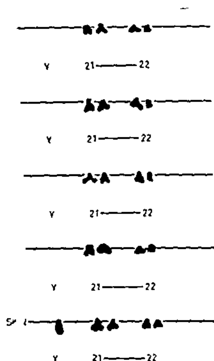


Fig 2 Partial karyotype of G group chromosomes from bone marrow cells (2n 45 XO 22q) and from the skin (2n 46 XY) of case 1

In case 2, the initial bone marrow examination showed a 2n 45 XO karyotype (fig 3) and similar observations were made on 3 subsequent occasions (table I) A phytohaemagglutinin-stimulated 72 h peripheral blood lymphocyte preparation examined shortly after presentation and again in October 1973 showed a normal karyotype 2n 46 XY The Philadelphia chromosome was not demonstrated in any of these preparations Fluorescent staining confirmed the absence of the Y chromosome in cells obtained from the bone marrow of both patients A fibroblast culture from a skin biopsy showed a normal 2n 46 XY karyotype in case 1 (table I)

Table 1 Chromosome counts and karyotype analysis in two patients with chronic granulocytic leukaemia and absence of the Y chromosome

	Date	Chromosome counts					Chromosomes absent in cells with 45 chromosomes							Karyotype
		44	44	45	46	47	A	B	C	D	E	F	G/Y	
Case 1														
Marrow	18 10 72	1	2	6									6	2n 45 XO 22q
Marrow	16 1 74		2	10									10	2n 45 XO 22q
Marrow	27 3 74	2	9	35	2	2							35	2n 45 XO 22q
Skin	5 4 74		5	4	21		1	1	1				1	2n 46 XY
Marrow	1 5 74		3	10	1								10	2n 45 XO 22q
Case 2														
Marrow	1 12 71		3	11	2								11	2n 45 XO
Blood	15 12 71	2		2	2							1	1	2n 46 XY
Marrow	25 7 73	4	4	32									32	2n 45 XO
Marrow	3 10 73	1	2	12									12	2n 45 XO
Blood	3 10 73		2	3	4		1	1		1				2n 46 XY
Marrow	22 10 73	3	3	23									23	2n 45 XO

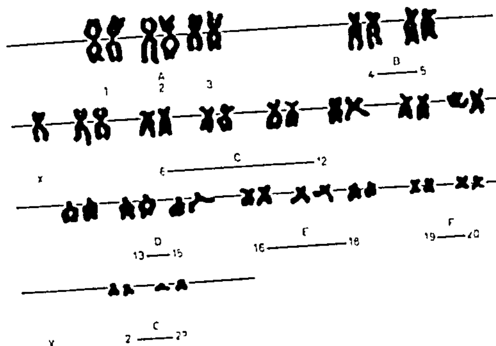


Fig 3 Chromosomal karyotype from a bone marrow cell (case 2)

Discussion

Absence of the Y chromosome in CGL is regarded as a good prognostic feature compatible with long survival [7] and SANDBERG and SAKURAI [18] have suggested that this abnormality prevents cells from entering the blast phase Several patients [2, 5, 8, 14, 20] have survived for over 8 years without transformation to the blast cell phase occurring and have given support to the view that absence of the Y chromosome is associated with a benign course However, this idea has been challenged recently [13] by a report of 2 patients with CGL and absence of the Y chromosome, who had a very short chronic phase of less than 18 months

Both our patients underwent a rapid blast cell transformation, case 1 *after 16 months and case 2 after 17 months in the chronic phase of* CGL. Apart from the XO state, case 1 had typical CGL and the haematological blast cell crisis occurring after a short chronic phase adds evidence for the view that absence of the Y chromosome does not necessarily protect against blast cell transformation Similar to a previously reported patient [21] case 2 was Ph¹-negative, but the clinical and haematological findings and the patient s response to treatment were consistent with CGL Ph¹-negative CGL is associated with a less favourable prognosis than the Ph¹ positive disease [7] and therefore this patient s short course should not necessarily be attributed to absence of the Y chromosome The terminal illness was characterised by tissue infiltration with primative myeloid cells and although unusual, similar lymphoid infiltrates have been reported previously [3 10, 11]

Indirect evidence for the association of the absent Y chromosome with the CGL clone is provided by the karyotypically normal lymphocyte preparations of case 2 and the normal skin culture of case 1, a finding noted in other patients [6 8]

Although previously reported patients with CGL lacking the Y chromosome have been phenotypically normal males, the majority have either been unmarried or have not produced children Unfortunately, we did not investigate the infertility of case 2 further Our first patient had 2 normal offspring, one son and one daughter, and in this respect was similar to patients previously reported [2 20]

Acknowledgement We wish to thank Dr K. M Lawrence Department of Child Health Welsh National School of Medicine for his helpful advice and for reviewing the manuscript

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Simultaneous Occurrence of Hypocholesterolemia, Hypocalcemia and Hypofibrinogenemia in a Case of Multiple Myeloma

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Key Words Hypocalcemia Hypocholesterolemia Hypofibrinogenemia Mel phalan Multiple myeloma

Abstract Hypocholesterolemia hypocalcemia and hypofibrinogenemia were si multaneously found in a patient with multiple myeloma An objective remission of the myeloma during Melphalan treatment was accompanied by a normalization of these three parameters

Abnormal values for several biological parameters including serum cholesterol, serum calcium and plasma fibrinogen, are not rarely found in patients with multiple myeloma [10] The occurrence of these abnormalities in one and the same patient is however exceptional We want to report a case of multiple myeloma presenting a low serum cholesterol and calcium level and hypofibrinogenemia at the same time

Case Report

A 48 year-old man was admitted to the hospital in December 1973 with general complaints of anorexia fatigue and intermittent pain in the right hypochondrium Some weks before macroscopic hematuria was present There was no history of hepatic disease and no alcohol abuse

Physical examination on admission showed an apparently ill but well nourished patient Pulse rate 76 min blood pressure 120 65 mm Hg The liver was enlarged and painful the edge could be felt 3 fingers below the right costal margin No splenomegaly was found The spine and kidneys showed no pressure tenderness Spi ders were noted on the thoracic cage and upper extremities as well as palmar ery themia Some purpuric spots were observed on the lower extremities

Urine negative for protein and glucose sediment unremarkable Hematocrit 28%, hemoglobin 8 1 g 100 ml white blood cell count 4 700 / l with 3% eosinophils, 3% stabs 47% mature neutrophils, 25% lymphocytes 11% monocytes and 9% atypical plasmocytes Platelet count 24 000 µl Erythrocyte sedimentation rate 136 mm in the first hour Total serum protein 12.3 g 100 ml with 63% in the γ globulin region on paper electrophoresis and only 27% in the albumin fraction Serum albumin level 3.3 g 100 ml Agarose gel electrophoresis showed an extrafraction in the serum IgA area but no Bence Jones protein in urine Serum IgG 320 mg 100 ml IgM <24 mg 100 ml and IgA 6 720 mg 100 ml

Serum bilirubin 1 mg 100 ml glutamic oxalacetic transaminase 37 Karmen U glutamic pyruvic transaminase 47 Karmen U and alkaline phosphatase 7.3 KAU The BSP clearance showed a half life of 9 6 min and 24 4% retention at 45 min Hepatitis B antigen was not present Total cholesterol 45 mg 100 ml Blood urea 45 mg 100 ml serum creatinine 1 2 mg 100 ml creatinine clearance 122 ml min Serum calcium 6 mg 100 ml and phosphorus 5.2 mg 100 ml The urinary calcium excretion varied between 100 and 180 mg/24 h Blood pH 7 40 plasma bicarbonate 24 mEq l An electrocardiogram demonstrated no abnormalities

The bone marrow showed a marked infiltration by plasma cells Their percentage ranged from 70 to 100% A considerable pleomorphism with variation in cell size number of nuclei and degree of nuclear immaturity was present

Skeletal X ray studies disclosed no abnormalities

Table 1 Follow up of some biological parameters

	Before treatment	After 2 weeks of treatment	After 1 month of treatment	After 4 months of treatment	After 6 months of treatment
Serum protein g 100 ml	12 3	9 1	10 2	7 8	6 9
Serum albumin %	27	31	32	51	60
γ-Globulins, %	63	57	54	32	15
IgA mg 100 ml	6 720	8,370	6 400	2 960	978
Serum cholesterol mg 100 ml	45	70	95	145	210
Serum calcium, mg 100 ml	6	8 1	8 5	9 5	9 3
Plasma fibrinogen mg 100 ml	95	95	105	170	375
Melphalan	10 mg daily		3 × 5 mg weekly		
Prednisolone	40 mg daily		30 mg daily	20 mg daily	

A coagulation study revealed a low fibrinogen level of 95 mg/100 ml, a prolonged activated thromboplastin time of 57.5 sec (normal <50). Quick value corresponding with 45%. The thrombin time was normal. Coagulation factors: F II 30%, F V 45%, F VII X 55%, F X 45%, F VIII 94%, F IX 65%. Serum fibrin/fibrinogen degradation products measured by hemagglutination inhibition immunoassay were not increased. The plasminogen level was decreased to 1.2 Remmert Cohen units (normal values: 2–3 RCU).

Treatment with Melphalan and corticosteroids (table I) resulted in a slow but continuous improvement with disappearance of the asthenia and of the hemorrhagic tendency. Simultaneously, a regression of the biological abnormalities occurred during that period, compatible with a gradual remission of the disease (table I).

Only 10% of myeloma cells were found in a bone marrow examination after 6 months of treatment.

The hepatomegaly decreased gradually, and after 6 months of treatment the edge of the liver could hardly be felt below the right costal margin. An improvement of the BSP clearance was noted with a half life of 7 min and a retention of 11% at 45 min.

Discussion

Lipid disturbances with hypocholesterolemia are frequently found in patients with multiple myeloma [2, 5, 7, 8]. It has been demonstrated that myeloma proteins may display antibody-like activity against β-lipoproteins. This mechanism can lead to an immunoelimination of β-lipoproteins [4–6]. A striking relationship between the intensity of a low serum cholesterol level and the height of the myeloma peak has been reported by several authors [2, 5, 7, 8]. A normalization of serum cholesterol is usually obtained if a significant decrease of the myeloma immunoglobulin level occurs during treatment [8] as in our patient.

Hypercalcemia is more common than a low serum calcium level in multiple myeloma [1]. Renal failure can be excluded in our patient as a cause of hypocalcemia. We think that the low serum albumin level can only be partially responsible for the low serum calcium, but we have no other explanation. DRIVSHOLM [1] did not found any relationship between the serum calcium and the serum albumin concentration in two myeloma patients with hypocalcemia. As for the cholesterol level, the serum calcium concentration normalized during treatment.

No increase of serum fibrin/fibrinogen degradation products could be demonstrated on different time intervals. Disseminated intravascular coagulation with secondary fibrinolysis or a primary fibrinolytic mechanism can be excluded [3]. The presence of a circulating anticoagulant with an

antithrombin activity can also be eliminated as the thrombin time was completely normal [9]. A decreased synthesis of fibrinogen in the liver is highly probable. An argument can be found in the decreased levels of different coagulation factors. Our patient presented also clinical and biological signs of hepatic disease. Unfortunately, the presence of a hemorrhagic tendency excluded the performance of a peritoneoscopy with liver biopsy. The regression of the clinical and biological signs of hepatic disorder during Melphalan therapy, however, pleads for a plasmocytic liver infiltration.

It should be stressed that, as demonstrated in table I, the hypocholesterolemia, hypocalcemia and hypofibrinogenemia occurred simultaneously in our patient and normalized as the treatment of the multiple myeloma resulted in a significant decrease of the IgA peak.

References

1 [illegible] A. Myel[illegible] Acta med. scand. [illegible]
2 [illegible]
3 [illegible]
4 [illegible]
5 [illegible]
6 [illegible]
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[illegible] Department of [illegible]

Book Reviews · Buchbesprechungen · Livres nouveaux

Theodore H. Spaet (ed.) **Progress in Hemostasis and Thrombosis, vol. 2.** Grune & Stratton, New York 1974. 373 pp., US $ 22.50 £ 10.80

The second volume of 'Progress in Hemostasis and Thrombosis' gives exhaustive reviews on very actual subjects all very different from each other, from the vascular intima components to properties of factor VIII, from pharmacodynamics of oral anticoagulants to a discussion on the bleeding time. All chapters are completed by an important list of references. Reviews dedicated to methods such as the chapter on bleeding time in this issue should be seriously considered for the future issues.

I find the second volume quite interesting, worth reading. It is the source of a very rich information also because of the variety of subjects. The only minor criticism which can generally be made is sometimes the absence of very precise data which must be looked for in the original literature. This book is not for beginners or students and requires basic knowledge. Reading of the second volume of 'Progress in Hemostasis and Thrombosis' can be warmly recommended. This volume is dedicated to Armand J. Quick, who with his prothrombin time, the first quantitative assay system of blood coagulability, gave a strong impulse to coagulation research.

I. Duckert, *Basel*

R. Harris, P. Allin and D. Viza (eds.) **Cell Differentiation.** Munksgaard, Copenhagen 1972. 350 pp. DKr 195.–

Der Inhalt dieses Symposionbandes ist heute so aktuell wie damals: wie sind die molekularen Mechanismen strukturiert und kontrolliert, welche die Zelldifferenzierung bestimmen? Dementsprechend liegt das Hauptgewicht des Symposions mit 26 Vorträgen bei der «Cell Biology Section» und einer recht informativen Panel-Zusammenfassung durch P. I. Allin. Hier geht es um die Organisation des genetischen Materials einschliesslich der Chromosomenstruktur, um die genetische Programmierung und um die möglichen biochemischen Mechanismen der zellulären Differenzierung. Die vorgeschalteten embryologischen, immunologischen und kanzerologischen Sektionen bringen viele Details, z.B. über die Mechanismen der Zellbewegung, der zellulären Metaplasie, der immunologischen Toleranz oder der Beziehung zwischen Differenzierung und Krebs. Dieser weitgespannte Rahmen sichert dem Band einen breiten Leserkreis. Ausstattung und Bildreproduktionen sind sehr gut.

I. Grundmann, *Münster*

5th IAEA Symposium

Los Angeles, Calif., October 25–29, 1976

Main topic: Medical radionuclide imaging.
Organizers: International Atomic Energy Agency, PO Box 590, A-1011 Vienna, Austria.
Scientific secretaries: L. J. JOHANSSON and T. MUNKNER, Medical Applications Section.

This Symposium will cover all aspects of radionuclide imaging techniques and their applications in clinical medicine and research. It will place emphasis on recent advances in methods and techniques, particularly as regards the development and refinement of instrumentation, the use of computers for data processing and analysis, and the introduction of new radiopharmaceuticals. It will also consider the results of radionuclide imaging in relation to those of other imaging techniques.

Further information and forms to accompany abstracts of papers intended for presentation at the Symposium may be obtained from national authorities for atomic energy matters. Abstracts must be submitted through these authorities so as to reach the International Atomic Energy Agency before May 31, 1976.

Factor VIII, an Announcement

A task force has recommended to the International Committee on Thrombosis and Haemostasis the adoption of a new nomenclature for the factor VIII-related activities. An 'on the line' system of nomenclature has been recommended for the three major classes of activities related to blood coagulation factor VIII, i.e. VIII C for the coagulant activity, VIIIR AG for the antigenic activities related to factor VIII, and VIIIR WF for the Von Willebrand factor activities related to factor VIII. Adoption of these recommendations, which are at present before the International Committee, will be deferred for at least 1 year while the task force receives and considers suggestions for modification. Anyone who wishes a copy of the full report or wishes to make suggestions to the task force should contact its chairman, Prof. JOHN B. GRAHAM, Department of Pathology, School of Medicine, University of North Carolina, *Chapel Hill*, NC 27514 (USA).

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Bearbeitet von G. Boehm, Basel
(B) = Book Reviews – Buchbesprechungen – Livres nouveaux
(C) = Communications – Vorträge – Communications

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